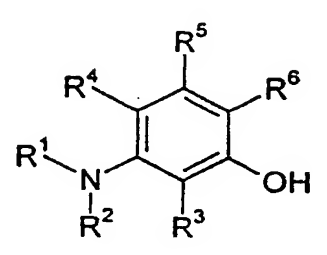


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C09B	A1	(11) International Publication Number: WO 99/07793 (43) International Publication Date: 18 February 1999 (18.02.99)
<p>(21) International Application Number: PCT/GB98/02334</p> <p>(22) International Filing Date: 4 August 1998 (04.08.98)</p> <p>(30) Priority Data: 9716476.8 4 August 1997 (04.08.97) GB</p> <p>(71) Applicant (for all designated States except US): NYCOMED AMERSHAM PLC [GB/GB]; Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): GRIFFITHS, John [GB/GB]; 9 Ashlea Close, Garforth, Leeds LS25 1JX (GB). MAMA, John [GB/GB]; 14 Lambton View, Harehills, Leeds LS8 5PE (GB). MILLAR, Valerie [GB/GB]; 81 Braziers Quay, South Street, Bishop's Stortford, Hertsfordshire CM23 3YW (GB). BRIGGS, Mark, Samuel, Jonathan [GB/GB]; 109 Park Place, Amersham, Buckinghamshire HP6 6NQ (GB). HAMILTON, Alan, Lewis [GB/GB]; Ashdown, 15 Church Grove, Little Chalfont, Amersham, Buckinghamshire HP6 6SH (GB).</p> <p>(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).</p>		<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
<p>(54) Title: DYE INTERMEDIATE AND METHOD</p> <p>(57) Abstract</p> <p>Various classes of dyes are provided having acid, ester or amide groups for covalent linking to biomolecules. The dyes may be prepared by use of a compound of formula (I) where R¹ comprises a linker and a carboxy including acid, salt, ester including N-hydroxysuccinimide, activated ester or amide group; R², R³, R⁴ and R⁵ are H, C₁-C₁₀ alkyl or aralkyl or a group to modify solubility or electronic or spectral properties or a functional linking group; or R⁴-R⁵ and/or R²-R⁴ and/or R²-R³ are linked to form an extended ring system; and R⁶ is H or CHO or NO.</p> <div style="text-align: right;">  <p>(I)</p> </div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

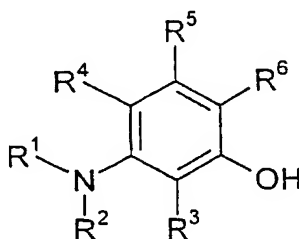
DYE INTERMEDIATE AND METHOD

5 Dyes have applications in many technologies, both new and well established, which count amongst them textile dyeing, optical data storage media and various detection methods, for both chemical and biological use. Within these applications various properties of the dyes are exploited in their actual detection which may ultimately be traced back to
10 absorption, reflectance and luminescence (chemiluminescence, fluorescence and phosphorescence). However, a recurring theme of many applications is the need to form a relatively permanent link, in the form of a covalent bond, between the dye and the substrate of choice. The substrate may be *inter alia*: a relatively inert surface or probe possibly of insoluble
15 polymeric origin; a macromolecule of biological origin e.g. a protein, antibody or oligonucleotide; or a small molecule of either biological or synthetic origin e.g. a hapten for use in directed or molecular recognition based applications; or a molecule which may modify the properties of the dye e.g. a water solubilising group such as a carbohydrate or other polar
20 residues; or a species which protects the dyes from or decreases the rate of photofading of the dye, such as hindered amines and various transition metal complexes; or possibly another dye conjugate system thus enabling the possibility of energy transfer or fluorescence quenching based applications. The requirement for a covalent linkage means that many
25 commercial products, spanning a large range of dye classes and possessing the desired spectral properties, cannot be used. This therefore necessitates either the modification of an existing product or the complete synthesis from appropriate materials such that a suitable grouping is available for linking the dye and substrate. There are many different sorts
30 of linkage known, the main requirements being that the linkage is relatively stable and that it is easily introduced in high yield or efficiency. One of the

- 2 -

most versatile groupings is that of the carboxylic acid which is readily converted to a range of reactive species or used to form a link to another substance containing the reactive linking species of choice.

This invention is concerned with a relatively simple and easily prepared dye intermediate and its use for preparing a range of dyes particularly fluorescent dyes having reactive groups by which they can be linked to various substrates. The intermediate is a m-aminophenol having the formula (I)



(I)

10

Where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N-hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

20

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

25

R^6 is H or $-CR^9O$ or N-O.

R^9 is H, $C_1 - C_6$ alkyl, phenyl or R^aR^c wherein R^a and R^c are

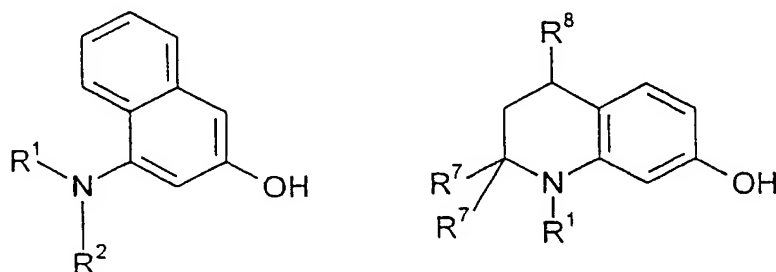
- 3 -

herein defined.

R^1 may be different or the same as R^2 .

R^2 , R^3 , R^4 and R^5 may contain carbon chains which are linked to form extended ring systems, either aliphatic or aromatic, for example

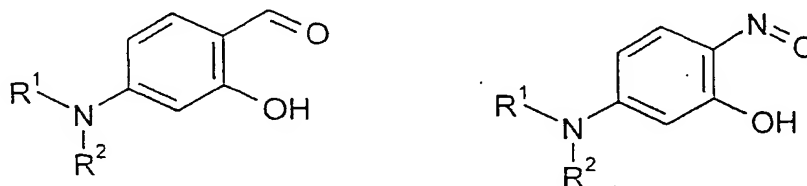
5



where each of R^7 and R^8 are defined as R^2 , R^3 , R^4 and R^5 .

Many of the dyes described below are made from compounds of formula (I) in which R^6 has been changed from H to either formyl or nitroso, for example

10



These may be made from the corresponding compounds where R^6 is H by standard methods.

15

Groups to modify solubility include phosphate, sulphonate, carbohydrate, poly(oxyethylene) and perfluoroalkyl. Groups to modify electronic and/or spectral properties include nitro, cyano, halogen and alkoxy. Functional groups for linking to another component include carboxylic acid or derivative or activated ester, azide, amine, hydroxyl, sulphonyl chloride, isothiocyanate, phosphoramidite, vinyl sulphone, maleimide, halotriazine, iodoacetamide and N – hydroxysuccinimide; see also Andrew Garman: "Non-Radioactive Labelling: A Practical

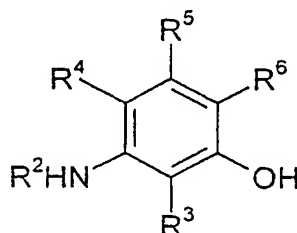
20

- 4 -

Introduction" published by Academic Press, London, 1997, and references cited therein.

These compounds may be linked, preferably through R^b or alternatively through R^2 , R^3 , R^4 , R^5 , R^7 or R^8 to a support such as
 5 polyethylene glycol, polyethyleneimine, polysaccharides such as dextran, or a derivatised substrate such as that used in solid phase synthesis e.g. polystyrene, polyacrylate or glass.

Preferably R^a is $C_1 - C_4$ alkylene. Certain of these compounds can be made by an advantageous process which forms
 10 another aspect of this invention, and which comprises reacting



with GG^1

15 where G is $CHR=CR-$ where R is H or $C_1 - C_4$ alkyl and G^1 is an electron withdrawing group for example CN or carboxy where the carboxy group is acid, salt, ester or amide.

Other compounds may be made by the use of $G^2R^aG^1$ where G^2 is a leaving group and R^a and G^1 are herein defined.

20 An intermediate compound of formula (I) may be immobilised on a support and there reacted to form the desired dye. This method may give access to a combinatorial library of dyes or an easy way of purifying dyes, or may permit the labelling of a first nucleotide or amino acid of a solid phase oligonucleotide or oligopeptide synthesiser.

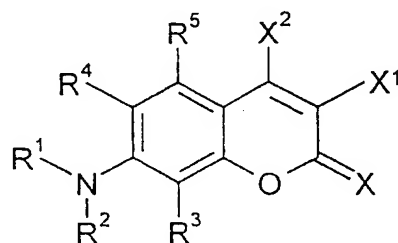
25 In one aspect, this invention provides use of the said intermediate to make a dye selected from a defined group, or a leuco-dye or reduced analogue of said dye. In another aspect, the invention provides

- 5 -

the resulting dyes, and their leuco-dye analogues, as new compounds.

The dyes are as follows (in each case, R^1 , R^2 , R^3 , R^4 and R^5 are as defined above for the intermediate compound (I); and may be the same or different at different parts of the molecule):

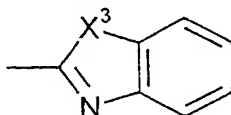
5



(II)

where X is O, NH or =NCOAr

X^1 is H, CN, CHO, $CH=N^+(R^dR^e)$, NO, COOH, COOR^f, CONR^gR^h, C₁ – C₁₀ alkyl, aralkyl or aryl or



10

where X^3 is NH, N-R^k, O or S, wherein X^1 is either unsubstituted or substituted by: a group to modify solubility; or a group to modify electronic and/or spectral properties; or a functional group for linking to another component,

15

R^d and R^e are alkyl, aryl, aralkyl,

R^f , R^g , R^h and R^k are R^aR^c wherein R^a and R^c are herein defined,

20

X^2 is H, CN, ORⁱ, Cl, Br, alkyl or aryl which maybe unsubstituted or substituted by: a group to modify solubility; or a group to modify electronic and/or spectral properties; or a functional group for linking to another component;

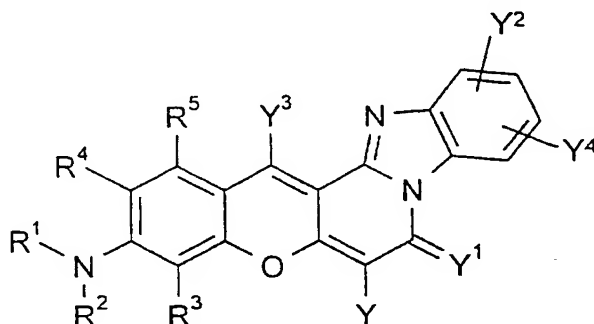
where Rⁱ is MeC₆H₄SO₂-, CH₃SO₂-, P=O(OR^j)₂

R^j is alkyl, aryl, aralkyl

- 6 -

X^1 and X^2 may contain atoms which are linked to form a carbocyclic, heterocyclic, aliphatic or aromatic ring system which may be substituted or unsubstituted with a group to modify electronic and/or spectral properties and/or a group to modify solubility properties and/or a functional linking group;

provided that, when X is O, then X^1 is not H, CN, or $C_1 - C_{10}$ alkyl, aralkyl or aryl; and provided that when X is O, then X^3 is not S;



(III)

where Y is CN or $CONH_2$ or CH_2NH_2 ,

Y^1 is NH or O or $=NCOAr$,

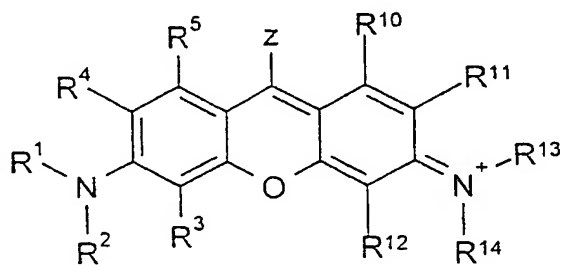
10

Y^2 and Y^4 are: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or

15

- R^aR^c wherein R^a and R^c are herein defined,

Y^3 is H or CN;



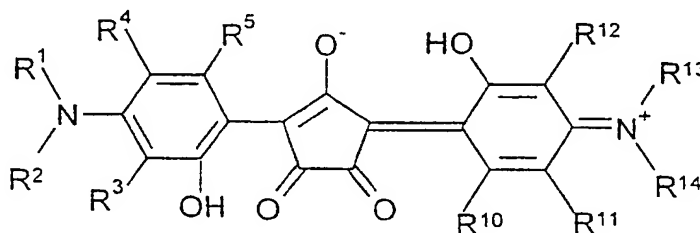
(IV)

- 7 -

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

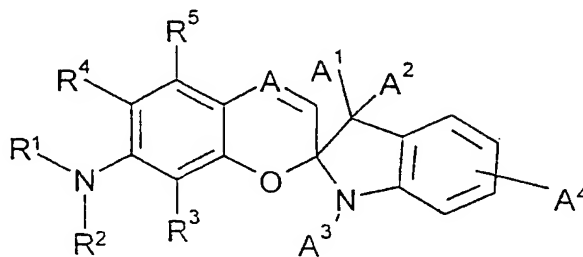
R^{10} and R^{11} ; and/or R^{13} and R^{11} ; and/or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

10 Z is aryl which is unsubstituted or substituted by: a group to modify solubility; or a group to modify electronic and/or spectral properties; or a functional group for linking to another component, provided that Z is not 2-carboxy-3,4,5,6-tetrachloro phenyl;



(V)

15 provided that $NR^{13}R^{14}$ is different from NR^1R^2 ;

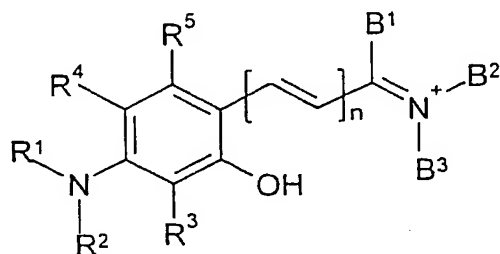


(VI)

where A is N or CH

20 A^1 , A^2 , A^3 and A^4 are the same or different and each is: H; or

C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or – R^aR^c where R^a and R^c are
 5 herein defined;



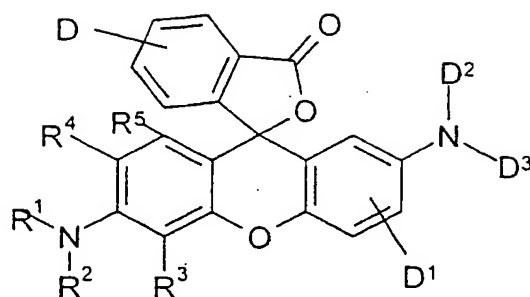
(VII)

where n is 0, 1 or 2, B¹ is H, C₁-C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to
 10 modify electronic and/or spectral properties, or a functional linking group, Cl, Br or F,

B² and B³ are the same or different and are selected from C₁-C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties,
 15 or a functional linking group,

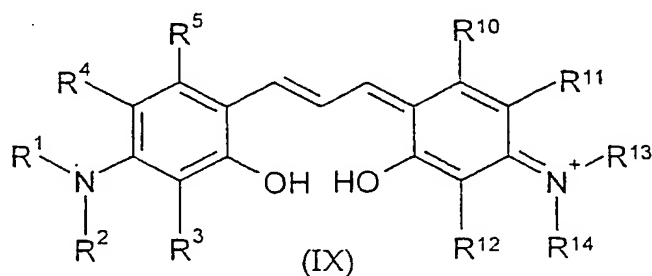
B¹ and B² and/or B¹ and B³ and/or B² and/or B³ may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted by a group to modify
 20 solubility, or a group to modify electronic and/or spectral properties, or a functional linking group;

- 9 -



(VIII)

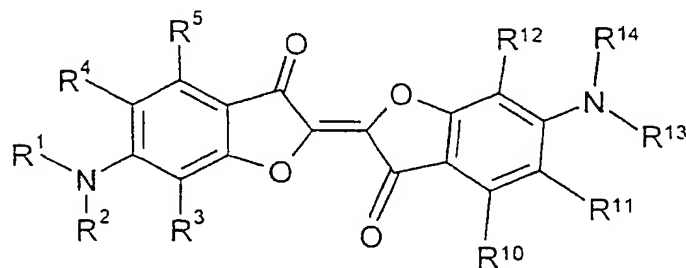
where D, D¹, D² and D³ are the same or different and each is:
 H; or C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen,
 nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is
 5 a group to modify solubility, or a group to modify electronic and/or spectral
 properties, or a functional linking group; or – R^aR^c where R^a and R^c are
 herein defined;



(IX)

10

and

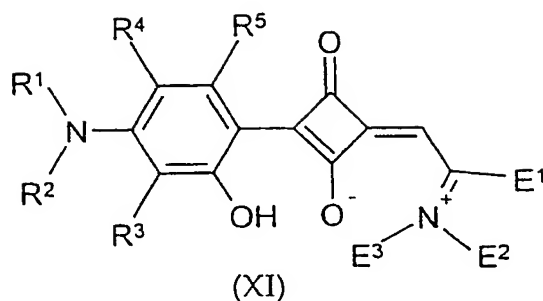


(X)

and

15

- 10 -

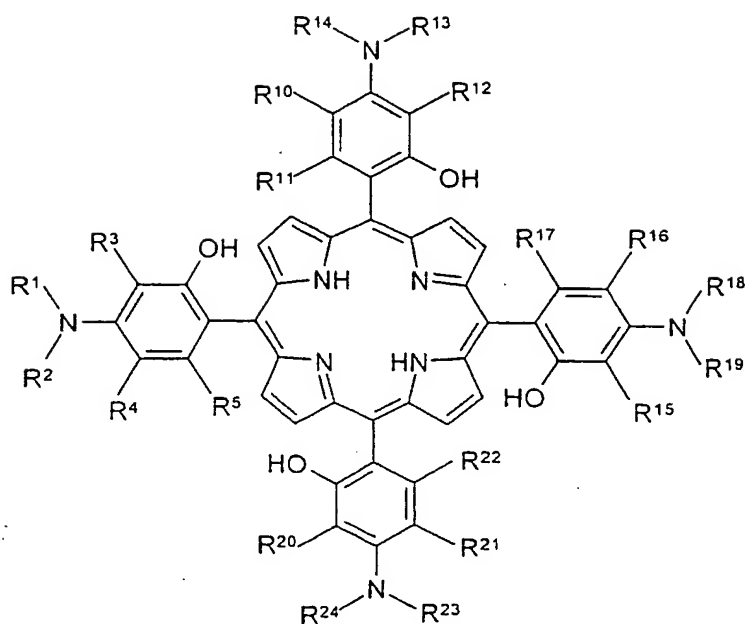


where E¹ is H, C₁-C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group, Cl, Br or F,

5 E² and E³ are the same or different and are selected from C₁-C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group,

10 E¹ and E² and/or E¹ and E³ and/or E² and/or E³ may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted by a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group;

- 11 -



(XII)

where R¹⁵, R¹⁶, R¹⁷, R¹⁸, R¹⁹, R²⁰, R²¹, R²², R²³ and R²⁴ are the same or different and each is: H; or C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or
 5 – R^aR^c wherein R^a and R^c are herein defined,

R¹⁶ and R¹⁷; and/or R²¹ and R²²; and/or R¹⁵ and R¹⁸ and/or R¹⁶ and R¹⁹; and/or R²⁰ and R²³; and/or R²¹ and R²⁴ may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic
 10 which is unsubstituted or substituted as above.

By virtue of the reactive group R¹, the dyes of the present invention may be combined with target materials to form conjugates. Suitable target materials may include antibodies, antigens, proteins, carbohydrates, polysaccharides, lipids, nucleotides, nucleic acids, polymer
 15 particles or surfaces and glass beads and surfaces. Thus, for example, the carboxyl group of R¹ may be converted to an N-hydroxysuccinimidyl ester for linking with an amino group of a polypeptide. These methods and the products resulting from them, either dye-labelled biomolecules or dyes

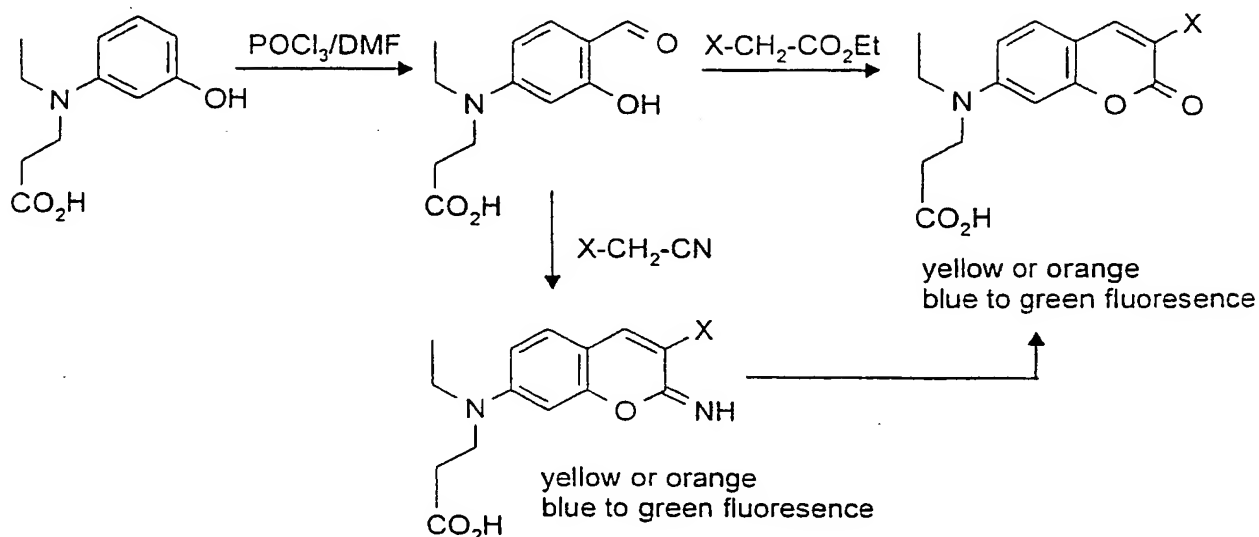
immobilised on particulate or massive surfaces, are envisaged as further aspects of the invention.

The main uses for the dyes of this invention are expected to be for labelling biologically active molecules such as nucleotides, oligo- and poly-nucleotides, nucleic acids and nucleic acid analogues (e.g. PNA), amino acids, peptides, proteins, antibodies, haptens, etc. for use in biological detection systems. Alternatively, the dyes may be conjugated to species which can direct the path of the dye within or aid entry to or exit from cells (live or dead); such as for example long alkyl residues to allow permeation of lipophilic membranes, or intercalating species to localise a dye in a nucleus or other cellular enclave containing double-stranded DNA or in vitro applications involving double-stranded nucleic acids. Dyes may also be immobilised on substrates such as particles or beads. The dyes may be involved in a resonance energy transfer system of the kind described in EPA 747 700.

The actual properties of the dyes used as detection labels vary. The observed property may simply be colour (based on light absorbance), luminescence or fluorescence (based on light emission) or other properties such as fluorescence lifetimes or degree of polarisation of emitted light. The dyes may also be used outside biological detection systems. For example, a dye may be immobilised on a probe for use in a chemical detection system e.g. a colour change, pH probe, or a chemical detector based on solvatochromism, electrochromism, thermochromism or photochromism. Reactive dyes may also be useful in printing e.g. in order to obtain improved substantivity effects.

There follow a number of example applications, which indicate a general chemical strategy by which intermediate compounds of formula (I) are used to make various families of dyes. The lettering system used is consistent within any particular application, but may not be consistent with that used in the introduction above and the claims below.

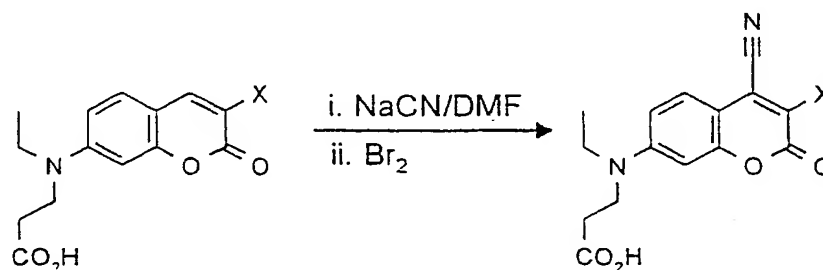
Application 1: Green-fluorescent coumarin dyes

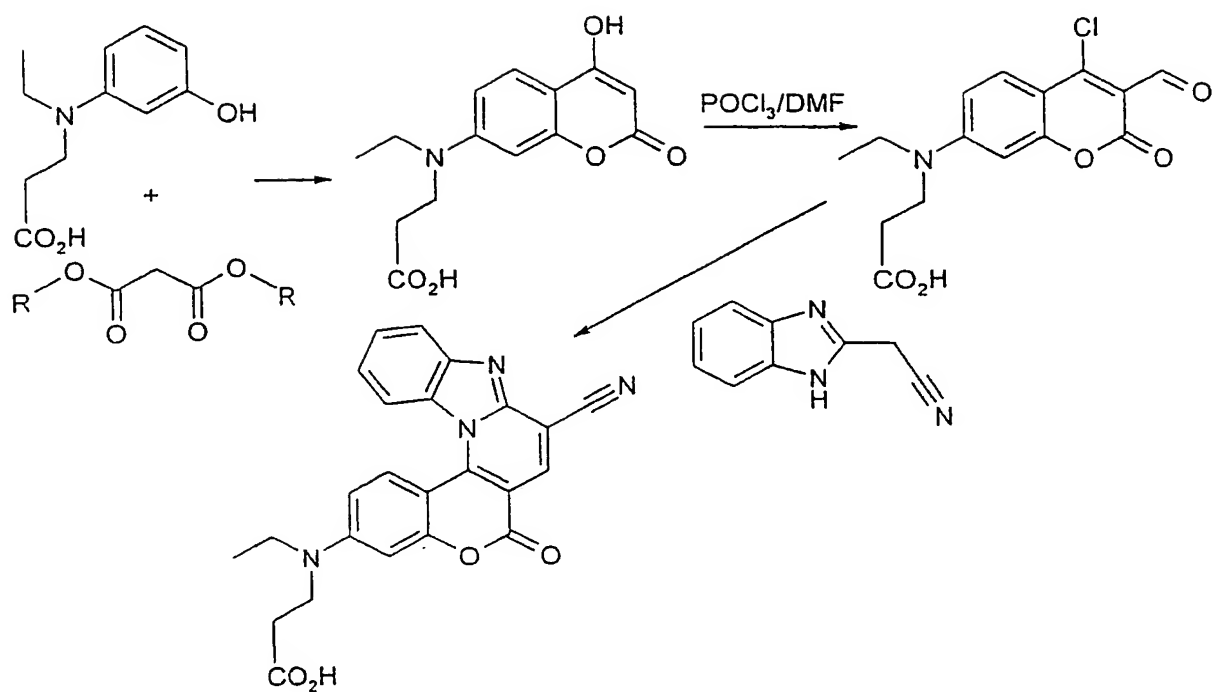


5 If X is simply -CN or an aryl substituent then the dyes are colourless with a blue fluorescence emission. To obtain yellow dyes with a green fluorescence the moiety X is a benzimidazole, benzoxazole or benzthiazole ring system, with or without additional substituents (e.g. halogen, sulphonic acid).

10

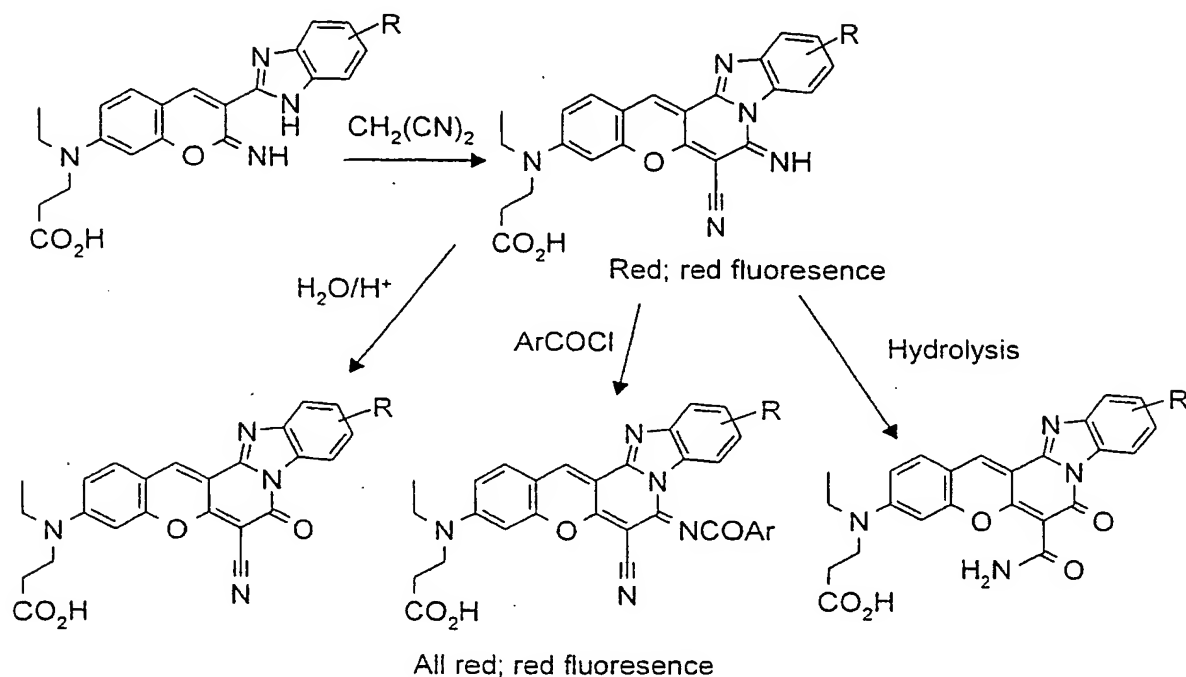
Application 2: Yellow and orange fluorescent coumarin dyes





- 15 -

Application 3: Red-fluorescent benzopyrano-benzimidazo-pyridines.

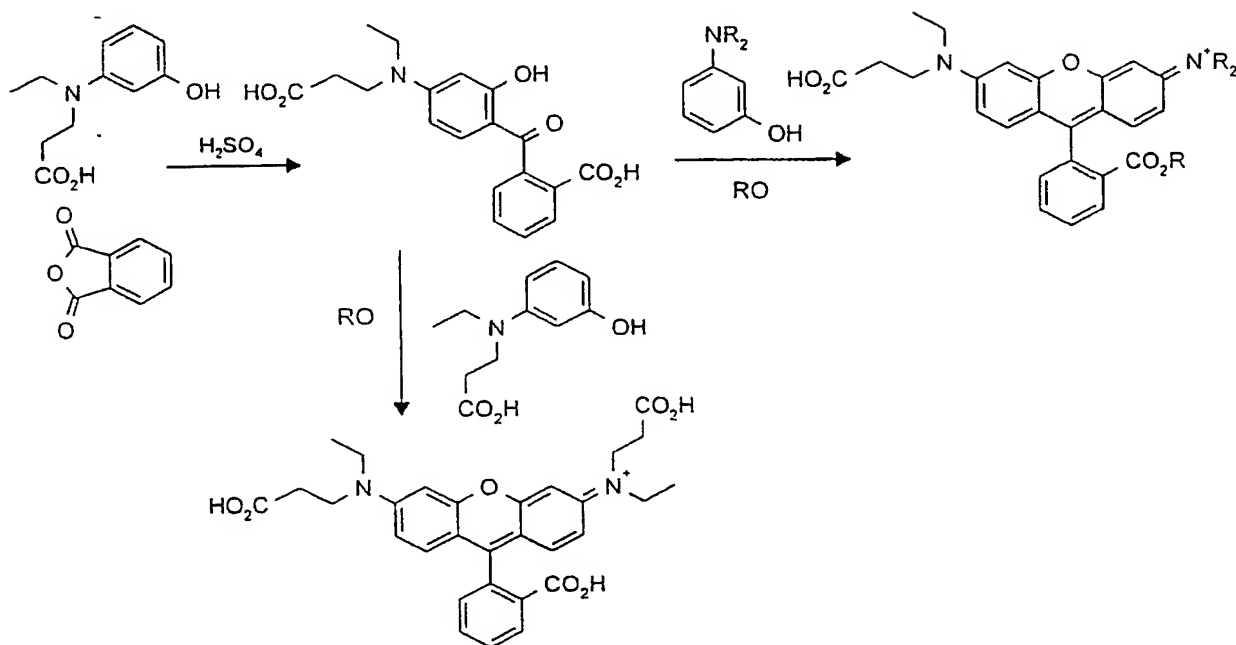


Substituents R in the benzimidazole ring may be H or any
 5 common substituent, but most usefully could be $-\text{SO}_3\text{H}$ to enhance water
 solubility or a grouping capable of forming a linkage, e.g. $-\text{CO}_2\text{H}$, to give a
 bis-functional dye.

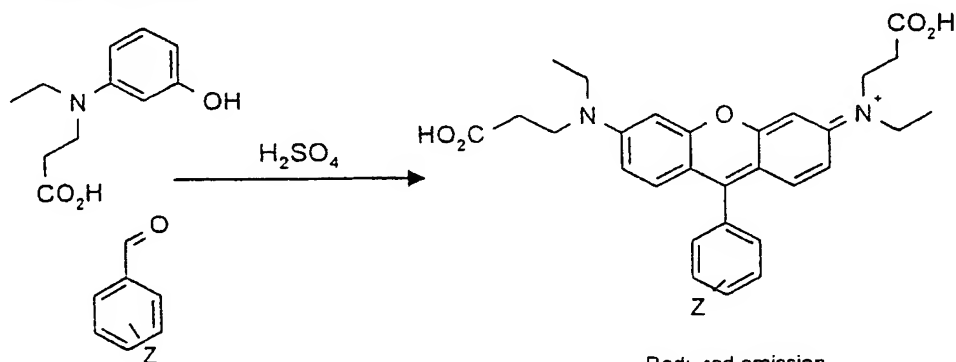
10

15

Application 4: Red-fluorescent rhodamine dyes.



As shown above, such dyes may be symmetrical or unsymmetrical. There is the problem of the additional carboxylic acid group in the *meso* phenyl ring, which should preferentially be esterified. Alternatively, sulphorhodamines or non-*meso*-carboxylated analogues could be considered:



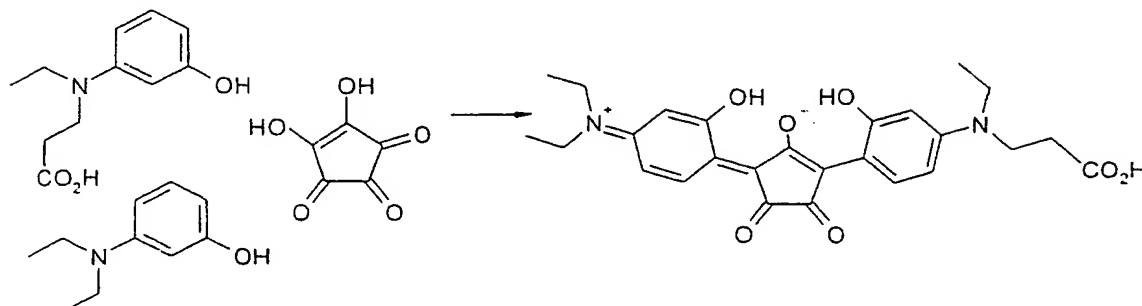
Red; red emission

Z = -SO₃H sulphorhodamines

10 Z = other substituent (e.g. halogen, alkoxy, alkyl, cyano).

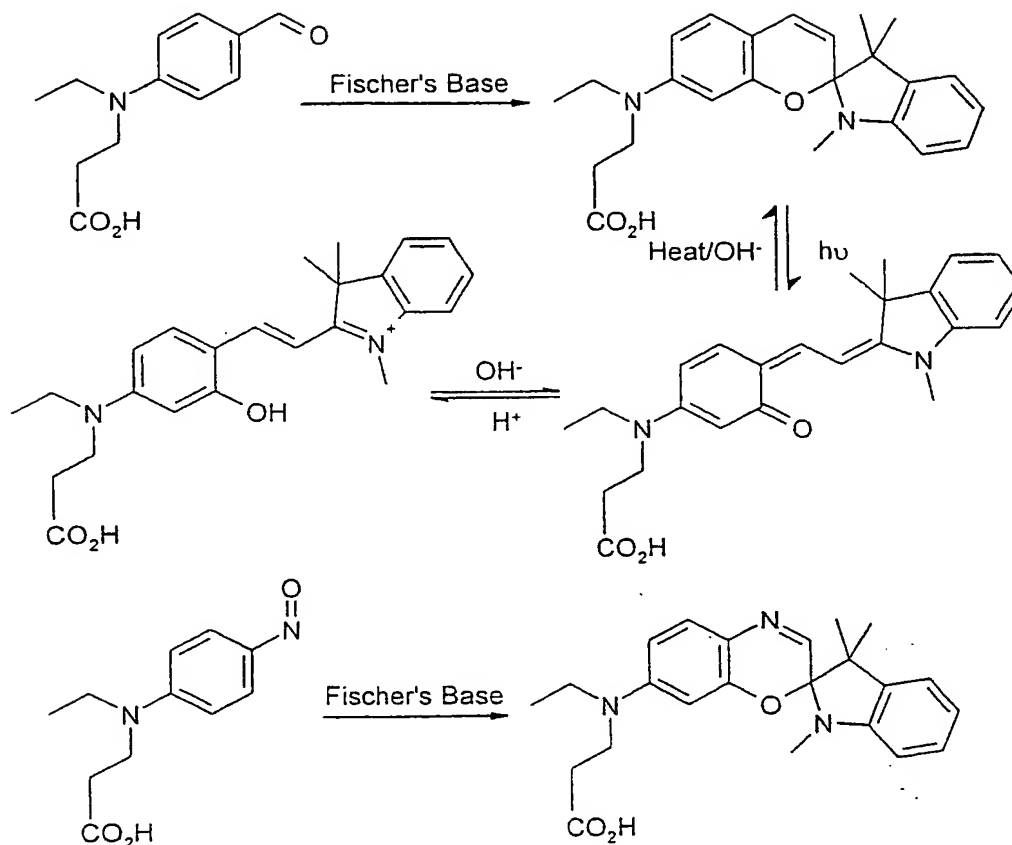
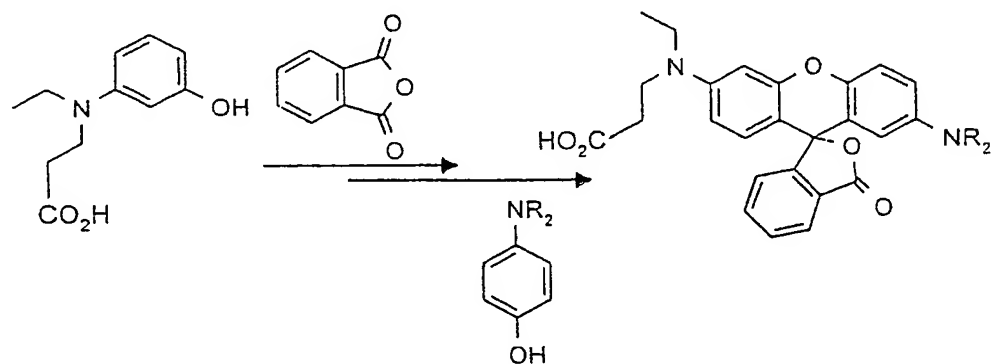
- 17 -

Application 5: Near-infrared absorptive croconium dyes



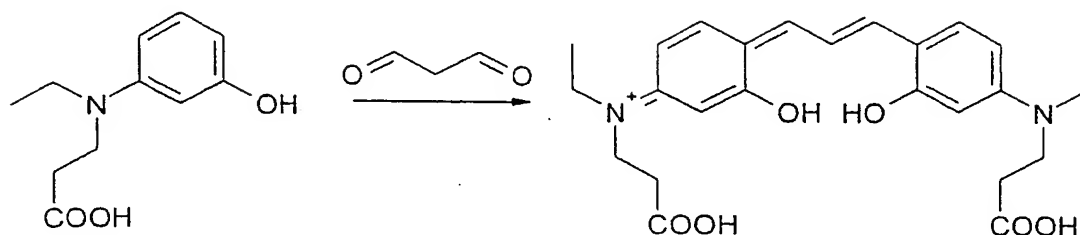
- 5 Use of this type of intermediate (*i.e.* with a *meta*-hydroxy group) is essential for the success of the reaction with croconic acid. If the dyes are fluorescent, then the emission will probably lie beyond the range of most fluorimeters, probably near 900nm.

- 18 -

Application 6: pH Sensitive Photochromic Dyes**Application 7: pH sensitive green-black fluorans**

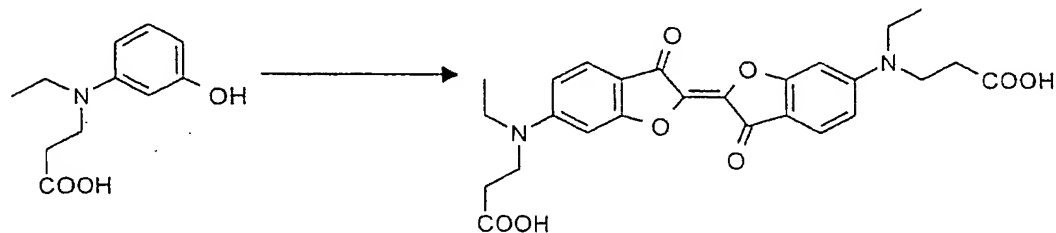
5

The open chain form of the dye is not fluorescent and is usually very dark in colour due to its multiple absorption band chromophore.

Application 8: Styryl *hemi*-cyanines.**Application 9: Leuco dyes for use as enzyme substrates.**

These are not dyes, as such, but a reduced, or leuco, form of those already listed which for example, may be chemically or enzymatically oxidised back to parent dye molecule. The dyes may be derivatised slightly to increase their stability.

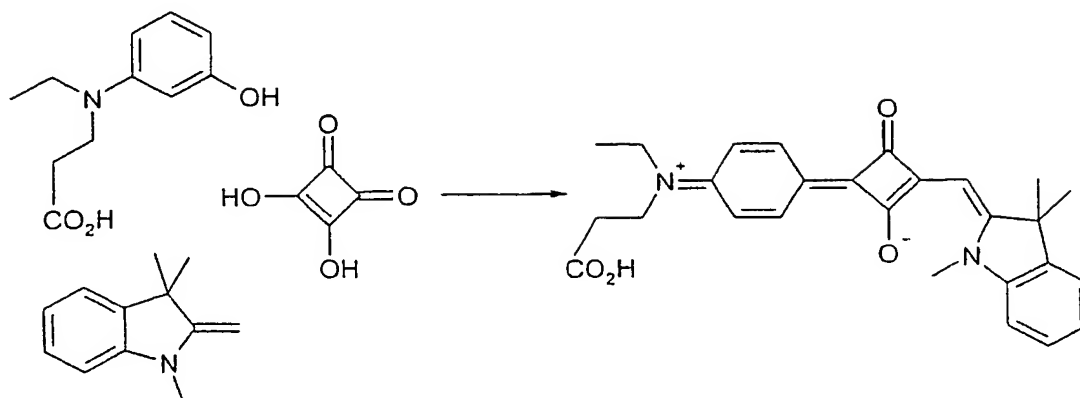
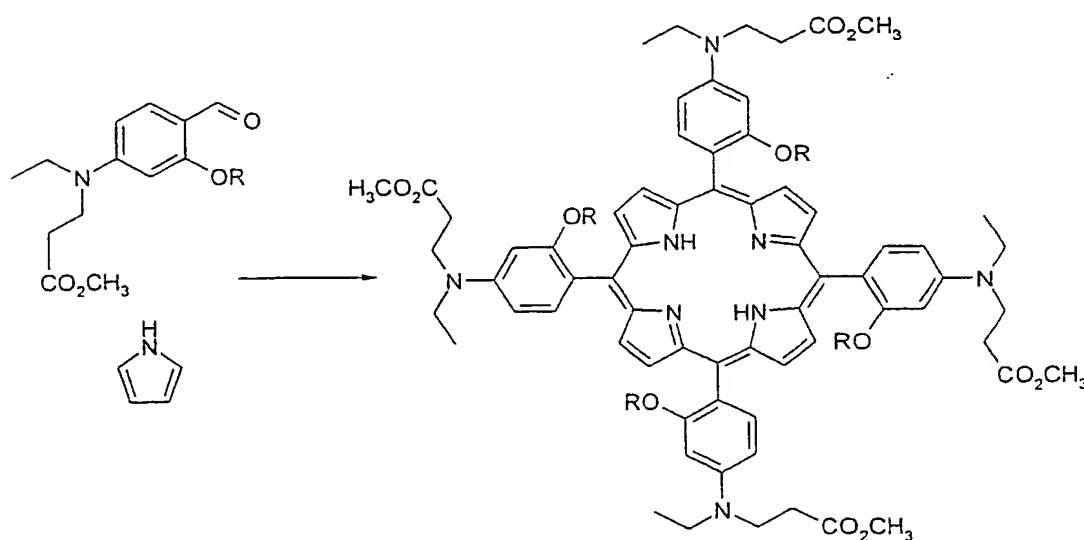
10

Application 10: Oxindigo derivatives

These dyes can have large Stoke's shift (50-100nm) emitting in the red, λ_{max} (Abs.) 510-530 nm, λ_{max} (Em.) 600-620 nm. They are also reported as being strongly fluorescent with high colour fastness. (Angew. Chem. Int. Ed. Engl. 1996, 35 (9) 1016), and therefore the ability to sum multiple scans.

20

- 20 -

Application 11: Squarylium Dyes**5 Application 12: 5,10,15,20-Tetraarylporphyrin Dyes**

with or without a central metal ion.

10 The following examples illustrate the invention. Examples 1 to 5, shows the preparation of compounds according to formula (I). Examples 6 to 18 show the use of intermediates of formula (I) in the preparation of various dyes.

Examples of Intermediate (I)

Example 1.

5 3-N-Ethyl-N-(2'methoxycarbonylethyl)-aminophenol (Ia)

(R¹= -CH₂CH₂CO₂Me, R²=Et, R³=R⁴=R⁵=R⁶=H)

3-Ethylaminophenol (5g, 36 mmol), methyl acrylate (10g, 120 mmol) and acetic acid (10ml) were heated at reflux for 5 hours. The excess methyl acrylate/acetic acid was then removed under reduced pressure at a
10 temperature no higher than 70°C. The residual oil was extracted into ethyl acetate (50ml), washed with saturated sodium bicarbonate solution (2x50ml), water (100ml), dried over MgSO₄, filtered and evaporated to dryness *in vacuo*. The product (Ia) was isolated as a brown oil (7.2g, 90%).

¹H NMR (300MHz, CDCl₃) 1.20 (t, 3H, J=6.9 Hz), 2.63 (t, 2H, J=7.1
15 Hz), 3.33 (q, 2H, J=6.9 Hz), 3.60 (t, 2H, J=7.5 Hz), 3.70 (s, 3H), 6.15 (d, 1H, J=5.4 Hz), 6.18 (s, 1H), 6.27 (d, 1H, J=5.4 Hz), 7.04 (t, 1H, J=6.9 Hz).

Example 2.

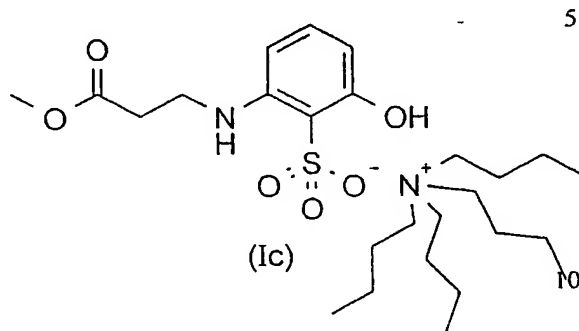
20

3-N-(2'Methoxycarbonylethyl)-N-ethylamino)-2-methylphenol (Ib)

(R¹= -CH₂CH₂CO₂Me, R²=H, R³=Me, R⁴=R⁵=R⁶=H)

3-Amino-2-methylphenol (1g, 8.1 mmol), methyl acrylate (0.73ml, 8.1mmol) and acetic acid (2ml) were heated together at reflux for 5 hrs and
25 then cooled to room temperature. The excess acetic acid was removed under reduced pressure and the residue purified by column chromatography (silica, 1:3 ethyl acetate: 40-60 petroleum ether). The product (Ib) was obtained as an off white solid (0.81g, 48%).

¹H NMR (300 MHz, CDCl₃) 1.98 (s, 3H), 2.66 (t, 2H, J=6.3 Hz), 3.49
30 (t, 2H, J=6.3 Hz), 3.71 (s, 3H), 6.25 (dd, 2H, J=7.8 Hz), 6.97 (t, 1H, J=8.1 Hz).

Example 3.**Tetra-n-butylammonium-6-hydroxy-2-(N-2'methoxycarbonylethyl-benzenesulphonate (Ic)**

2-Amino-6-hydroxybenzenesulfonic acid (236mg, 1.25mmol) and tetra-n-butylammonium hydroxide (1M solution in methanol, 1.25ml, 1.25mmol) were stirred together at room temperature for 1hr. The methanol was removed under reduced pressure to leave a yellow oil which was washed with ether (3x5ml). To the yellow oil was added methyl acrylate (0.5ml, 5.5mmol) and acetic acid (1ml) and the mixture heated at reflux for 48hrs. Excess methyl acrylate/acetic acid was removed under reduced pressure and the residue purified by column chromatography (silica: 20% methanol in ethyl acetate). The product (Ic) was obtained as an off white solid (50mg, 7.3%), partially in the form of the tetrabutylammonium salt.

¹H NMR (300MHz, d₆DMSO) 3.23 (t, 2H, J=5.7 Hz), 3.53 (t, 2H, J=5.7 Hz), 3.60 (s, 3H), 5.87 (s, 1H), 6.01 (d, 1H, J=6.3 Hz), 7.10 (d, 1H, J=8.4 Hz).

Example 4.**3-Acetoxy-N-ethyl-N-(2'methoxycarbonylethyl)-aniline (Id)**

(R¹= -CH₂CH₂CO₂Me, R²=Et, R³=R⁴=R⁵=R⁶=H, OCOCH₃)

3-N-Ethyl-N-(2'methoxycarbonylethyl)-aminophenol (Ia) (7.2g, 30 mmol) and acetic anhydride (10ml) were heated at 100°C for 4 hours. On

- 23 -

cooling the reaction mixture was poured onto ice and the pH adjusted to 4-5 with KOH solution (10%). The product was extracted into dichloromethane, dried over MgSO_4 , filtered and evaporated to dryness *in vacuo*. The product (Id) was isolated as a brown oil (7.4g, 93%).

5 ^1H NMR (300MHz, CDCl_3), 1.14 (t, 3H, $J=13.3$ Hz), 2.28 (s, 3H), 2.60 (t, 2H, $J=7.4$ Hz), 3.36 (q, 2H, $J=7.2$ Hz), 3.63 (t, 2H, $J=7.4$ Hz), 3.71 (s, 3H), 6.34 (s, 1H), 6.39 (d, 1H, $J=7.4$ Hz), 6.52 (d, 1H, $J=8.4$ Hz), 7.20 (t, 1H, $J=8.3$ Hz).

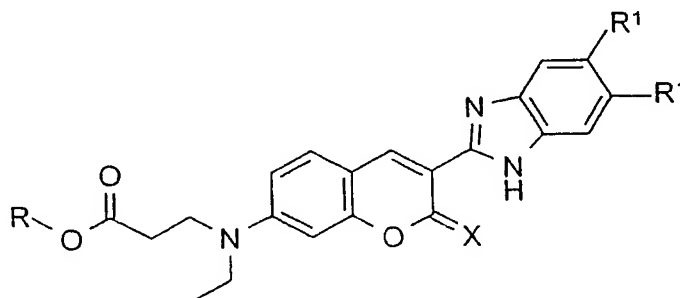
10 **Example 5.**

2-Acetoxy-4-(N-ethyl-N-(2'methoxycarbonylethyl)-aminobenzaldehyde
(Ie)

($\text{R}^1 = -\text{CH}_2\text{CH}_2\text{CO}_2\text{Me}$, $\text{R}^2 = \text{Et}$, $\text{R}^3 = \text{R}^4 = \text{R}^5 = \text{H}$, $\text{R}^6 = \text{CHO}$, OCOCH_3)

3-Acetoxy-N-ethyl-N-(2'methoxycarbonylethyl)-aniline (Id) (6.5g, 24
15 mmol) was dissolved in dimethylformamide (10ml, 0.13 mol) and cooled to below 5°C . Phosphorus oxychloride (3ml, 33 mmol) was added dropwise maintaining the temperature below 10°C . The reaction mixture was heated to 90°C for 1.5 hours and then cooled to room temperature and carefully poured onto ice (400g). The pH was adjusted to 5 with the addition of
20 saturated sodium acetate solution and stirred for 3 hours. The product was extracted into dichloromethane (2x150ml), washed with water (2x150ml), dried over MgSO_4 , filtered and evaporated to dryness *in vacuo*. The product was purified by column chromatography (silica; ethyl acetate) and isolated as a pale yellow oil (4.1g, 58%).

25 ^1H NMR (300 MHz, CDCl_3) 1.18 (t, 3H, $J=8.6$ Hz), 2.38 (s, 3H), 2.60 (t, 2H, $J=7.1$ Hz), 3.45 (q, 2H, $J=7.2$ Hz), 3.69 (t, 2H, $J=7.2$ Hz), 3.76 (s, 3H), 6.56 (s, 1H), 6.59 (d, 1H, $J=9$ Hz), 7.69 (d, 1H, $J=9$ Hz), 9.77 (s, 1H).

Examples of Green Fluorescent Coumarin Dyes (II) - Application 1.Example 6.IIa; R=Me, X=NH, R¹=HIIb; R=H, X=NH, R¹=HIIc; R=H, X=O, R¹=HIId; R=H, X=O, R¹=SO₃K

5

Benzimidazol-2'-yl-7-(N-ethyl-N-(2''methoxycarbonyl)ethyl)amino) imino
coumarin (IIa)

2-Acetoxy-4-(N-ethyl-N-(2'methoxycarbonyl)ethyl)-aminobenzaldehyde (Ie) (2.18g (60% purity), 4.5 mmol), 2-cyanomethylbenzimidazole (0.72g, 4.5 mmol), piperidine (0.45ml) and dimethylformamide (5ml) were stirred together at room temperature for 4 hours. Methanol (15ml) was added and the product filtered off, washed with methanol and dried. The product was isolated as a yellow solid (1.2g, 68%).

15 λ_{max} (DMF) 422nm.Fluorescence (DMF) λ_{ex} 422nm; λ_{em} 490nm; Stokes Shift 68nm.

¹H NMR (300mHz, d₆DMSO), 1.10 (t, 3H, J=7.1 Hz), 2.62 (t, 2H, J=6.9 Hz), 3.42 (q, 2H, J=6.6 Hz), 3.61 (s, 3H), 3.66 (t, 2H, J=6.6 Hz), 6.43 (s, 1H), 6.62 (d, 1H, J=8.7 Hz), 7.17 (m, 2H), 7.45 (d, 1H, J=8.7 Hz), 7.62 (broad s, 2H), 8.36 (broad s, 1H).

20

- 25 -

Benzimidazol-2'-yl-7-(N-carboxyethyl-N-ethylamino) imino coumarin
(IIb)

Ethanol potassium hydroxide (2ml (5%)) was added to a solution of the coumarin (IIa) (0.20g, 5.13mmol) in ethanol (10ml). The reaction mixture was heated at reflux for 10 mins at which point a further aliquot of 5% ethanolic KOH (4ml) was added and the mixture was heated at reflux for a further 15 mins. On cooling to room temperature the pH was adjusted to pH 5 with the addition of 10N hydrochloric acid. The ethanol was removed under reduced pressure and the residue dissolved in dichloromethane (20ml). The product was extracted into 1M NaOH solution (50ml) and then reprecipitated by adding 10N hydrochloric acid, the solid was filtered off, washed with water and dried. The product (IIb) was obtained as a bright yellow solid (90mg, 47%).

λ_{\max} (DMF) 425nm.

Fluorescence (DMF) λ_{ex} 425nm; λ_{em} 490nm; Stokes Shift 65nm.
FAB-MS m/z = found 399 (MNa⁺), 377 (MH⁺) [theoretical (C₂₁H₂₀N₄O₃) 376].

¹H NMR (300MHz, d₆DMSO), 1.13 (t, 3H, J=6.6 Hz), 2.57 (t, 2H, J=7.5 Hz), 3.53 (q, 2H, J=7.5 Hz), 3.69 (t, 2H, J=7.5 Hz), 6.70 (s, 1H), 6.84 (d, 1H, J=11.0 Hz), 7.16 (m, 1H), 7.24 (m, 1H), 7.61 (m, 1H), 7.64 (m, 1H) 7.72 (d, 1H, J=9.2 Hz), 8.93 (s, 1H).

Benzimidazol-2'-yl-7-(N-carboxyethyl-N-ethylamino) coumarin (IIc)

Imino coumarin (IIa) (0.5g, 1.28mmol), water (45ml) and conc. HCl (5ml) were heated at 100°C for 2 hours, over which time the dye dissolved and reprecipitated. The product was filtered off, washed with methanol and dried. The product was isolated as a bright yellow solid (0.3g, 63%).

λ_{\max} (DMF) 438nm.

Fluorescence (DMF) λ_{ex} 438nm; λ_{em} 495nm; Stokes Shift 57nm.

¹H NMR (300MHz, d₆DMSO), 1.10 (t, 3H, J=6.9 Hz), 2.57 (t, 2H, J=7.1 Hz), 3.52 (q, 2H, J=7.2 Hz), 3.68 (t, 2H, J=7.2 Hz), 6.70 (s, 1H),

- 26 -

6.84 (d, 1H, $J=11.4$ Hz), 7.16 (m, 2H), 7.60 (m, 2H), 7.73 (d, 1H, $J=9.3$ Hz), 8.96 (s, 1H).

3-(5',6'-disulphobenzimidazol-2'-yl-7-(N-carboxyethyl-N-ethylamino)

5

coumarin (IIId)

To coumarin (IIc) (90mg, 0.24mmol) was added chlorosulphonic acid (0.5ml). The reaction mixture was stirred at 40°C for 5 hrs, cooled, and then carefully quenched over ice. The orange precipitate was filtered off, washed with water, ethyl acetate and dried. The dye was purified by HPLC
10 (RP C₁₈; 80:20 water:ethanol). The product (IIId) was obtained as a orange solid (5mg, 3.6%).

λ_{\max} (1:1 MeOH:H₂O) 370nm.

Fluorescence; none detected.

¹H NMR (300 MHz, d₆DMSO) 1.21 (t, 3H), 3.4 (broad t, 2H), 3.61 (q, 2H), 4.04 (t, 2H), 6.91 (d, 1H), 7.59 (d, 1H), 7.70 (s, 2H), 7.98 (s, 1H),
15 8.92 (s, 1H).

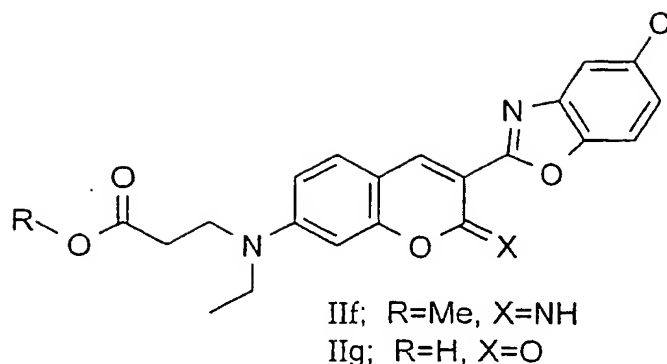
It was noted that if coumarin dye (IIId) was treated with methanol containing a catalytic quantity of sulphuric acid the non-fluorescent dye (IIId) was converted to the highly fluorescent dye (IIe);

20 λ_{\max} (1:1 MeOH:H₂O) 424nm.

Fluorescence (1:1 MeOH:H₂O) λ_{ex} 406nm; λ_{em} 480nm;

Stokes Shift 74nm.

- 27 -

Example 7.

3-(6'chlorobenzimidazol-2'-yl-7-(N-ethyl-N-(2''methoxycarbonylethyl)amino) imino coumarin - (IIIf)

2-Acetoxy-4-(N-ethyl-N-(2'methoxycarbonylethyl)-amino)-benzaldehyde (Ie) (1g , 3.4 mmol), 2-cyanomethyl-6-chloro-benzoxazole (0.66g, 3.4 mmol), piperidine (0.6ml) and dimethylformamide (2.5ml) were stirred together at room temperature for 5 hours. The crude dye was precipitated from the reaction mixture by adding ether and then filtered off, washed with ether and dried. The dye was purified by column chromatography (silica; 1:4 methanol:ethyl acetate). The product (IIIf) was obtained as an yellow/brown solid (0.5g, 36%).

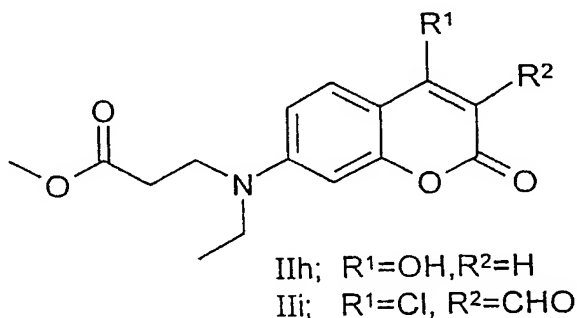
λ_{\max} (MeOH) 454nm.

Fluorescence (MeOH) λ_{ex} 453nm; λ_{em} 494nm; Stokes Shift 41nm.

^1H NMR (300MHz, d_6DMSO), 1.10 (t, 3H, $J=7.1$ Hz), 2.64 (t, 2H, $J=7.1$ Hz), 3.47 (q, 2H, $J=6.9$ Hz), 3.61 (s, 3H), 3.68 (t, 2H, $J=7.1$ Hz), 6.50 (s, 1H), 6.65 (d, 1H, $J=6.6$ Hz), 7.30 (d, 1H, $J=9.0$ Hz), 7.53 (d, 1H, $J=9.0$ Hz), 7.76 (d, 1H, $J=9$ Hz), 8.35 (s, 1H).

Examples of Yellow and Orange Fluorescent Coumarins Dyes (II) and Intermediates used in their preparation - Application 2.

5 **Example 8.**



7-(N-Ethyl-N-(2'methoxycarbonyl)ethyl)-amino)-4-hydroxy coumarin (IIh)

3-N-Ethyl-N-(2'methoxycarbonyl)ethyl-aminophenol (Ia), malonic acid bis-2,4,6-trichlorophenol ester and toluene (40ml) were heated at reflux for 4 hours. On cooling the product was filtered off, washed with cold acetone and dried. The product (IIh) was obtained as a pale yellow solid (4.1g, 63%).

¹H NMR (300 MHz, d₆DMSO) 1.07 (t, 3H, J=7.0 Hz), 2.60 (t, 2H, J=7.1 Hz), 3.43 (q, 2H, J=7.0 Hz), 3.60 (s, 3H), 3.62 (t, 2H, J=7.0 Hz), 5.26 (s, 1H), 6.49 (s, 1H), 6.67 (d, 1H, J=8.7 Hz), 7.54 (d, 1H, J=8.0 Hz).

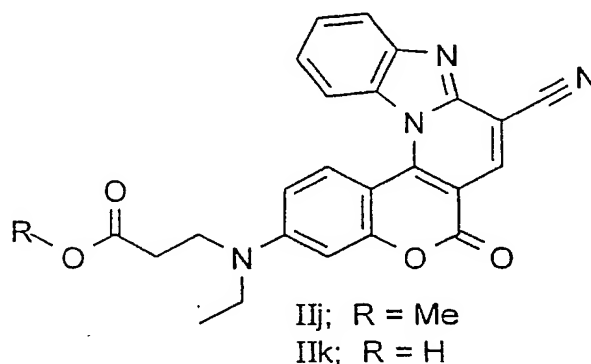
4-Chloro-7-(N-ethyl-N-(2'methoxycarbonyl)ethyl)-amino coumarin-3-carboxaldehyde (IIi)

7-(N-Ethyl-N-(2'methoxycarbonyl)ethyl)-amino)-4-hydroxy coumarin (IIh) (2.5g, 8.6mmol) and dimethylformamide (15ml) were cooled to below 5°C. Phosphorus oxychloride (1ml) was added dropwise maintaining the temperature below 10°C. The reaction mixture was gently warmed to 35°C for 3 hours. On cooling the reaction mixture was poured onto ice and stirred for one hour. The product was extracted into ethyl acetate (50ml), washed

with water (100ml), dried over MgSO_4 , filtered and evaporated to dryness *in vacuo*. The product was purified by column chromatography (silica; ethyl acetate). The product (IIi) was isolated as an orange solid (2.18g, 75%).

^1H NMR (300 MHz, d_6DMSO), 1.06 (t, 3H, $J=7.0$ Hz), 2.66 (t, 2H, $J=7.1$ Hz), 3.53 (q, 2H, $J=7.0$ Hz), 3.61 (s, 1H), 3.73 (t, 2H, $J=7.0$ Hz), 6.67 (s, 1H), 6.93 (d, 1H), 7.82 (d, 1H), 10.07 (s, 1H).

Example 9.



10

3-(N-ethyl-N-(2'methoxycarbonylethyl)-amino)-6-oxo- 6H-[1]Benzopyrano[3',4':5.6]pyrido[1.2a]benzimidazole-8-carbonitrile, (IIj)

Coumarin (IIi) (0.5g, 4.8mmol), 2-cyanomethylbenzimidazole (0.23g, 4.8mmol) and acetic anhydride (5ml) were heated together at 100°C for 4 hours. On cooling the product was precipitated out of the reaction mixture with ether. The product was filtered off, washed with ether and dried. The product (IIj) was obtained as an orange solid (0.5g, 74%).

λ_{max} (MeOH) 490nm.

Fluorescence (MeOH) λ_{ex} 490nm; λ_{em} 540nm; Stokes Shift 50nm.

20

^1H NMR (300 MHz, d_6DMSO), 1.16 (t, 3H, $J=6.8$ Hz), 2.70 (t, 2H, $J=6.9$ Hz), 3.56 (q, 2H, $J=6.9$ Hz), 3.63 (s, 3H), 3.76 (t, 2H, $J=7.4$ Hz), 6.81 (s, 1H), 6.88 (d, 1H, $J=9$ Hz), 7.41 (t, 1H, $J=7.8$ Hz), 7.63 (t, 1H, $J=7.5$

- 30 -

Hz), 7.98 (d, 1H, $J=8.1$ Hz), 8.13 (d, 1H, $J=8.4$ Hz), 8.15 (d, 1H, $J=9.6$ Hz), 8.51 (s, 1H).

3-(2-carboxyethyl-N-ethylamino)-6-oxo- 6H-

[1]Benzopyrano[3',4':5,6]pyrido[1,2a]benzimidazole-8-carbonitrile,

(IIk)

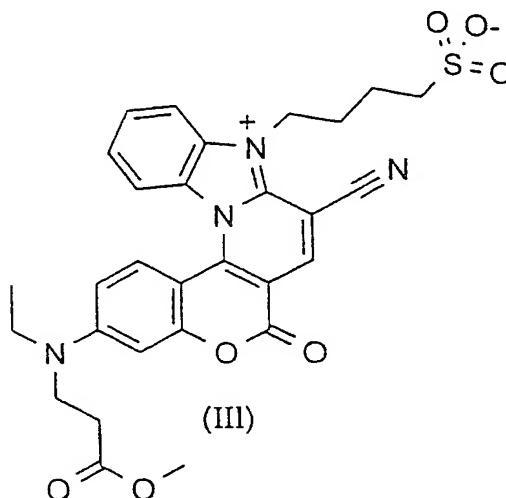
5

Coumarin (IIj) (50mg, 0.11 mmol) and ethanolic KOH (0.5ml (5%)) were heated at reflux for 10mins. On cooling the pH was adjusted to 7 with hydrochloric acid. The product was filtered off, washed with water and dried. The product was obtained as an orange solid (50mg, 106% - still
10 contaminated with inorganic salts).

λ_{\max} (MeOH) 456nm.

Fluorescence (MeOH) λ_{ex} 487nm; λ_{em} 540nm; Stokes Shift 50nm.

^1H NMR (300 MHz, d_6DMSO), 1.16 (t, 3H, $J=6.8$ Hz), 2.58 (t, 2H, $J=7.4$ Hz), 3.43 (q, 2H, $J=6.9$ Hz), 3.60 (t, 2H), 6.33 (s, 1H), 6.39 (d, 1H, $J=8.1$ Hz), 6.63 (d, 1H, $J=8.4$ Hz), 7.07 (d, 1H, $J=8.7$ Hz), 7.16 (t, 1H, $J=8.0$ Hz), 7.51 (t, 1H, $J=7.8$ Hz), 7.90 (d, 1H, $J=7.8$ Hz), 8.6 (s, 1H).
15

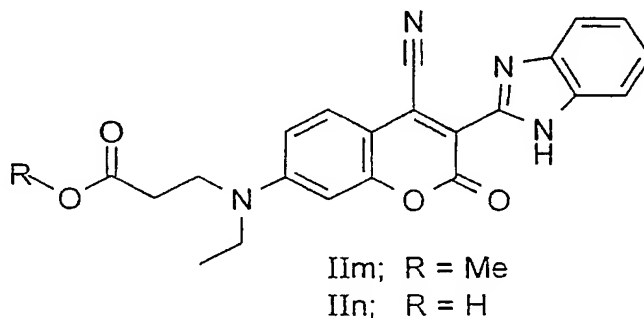


3-(N-ethyl-N-(2'methoxycarbonylethyl)-amino)-6-oxo- 6H-
[1]Benzopyrano[3',4':5,6]pyrido-3-sulphobutyl-[1,2a]benzimidazolium-
8-carbonitrile (III)

Coumarin (IIj) (50mg, 0.11 mmol); 1,4-butanedisulfone (17.5mg, 0.13 mmol)
 5 and butyronitrile (0.5ml) were heated together at reflux for 5 hours. On
 cooling the dye was precipitated out of the reaction mixture with ethyl
 acetate and filtered off. The dye was purified by preparative TLC (RP C₁₈;
 3:2 methanol:water). The product was obtained as an orange solid (16mg,
 25%).

10 MALDI-TOF-MS m/z =; found 576 (M); [theoretical (C₂₉H₂₈N₄SO₇)
 576]

Example 10.



15 3-(benzimidazol-2'-yl)-4-cyano-7-(N-ethyl-N-(2''methoxycarbonylethyl)
amino) coumarin - (IIIm) and 3-(benzimidazol-2'-yl)-4-cyano-7-(N-
carboxyethyl-N-ethylamino) coumarin (IIn)

Imino coumarin (IIa) (1g, 2.56 mmol) was dissolved in
 20 dimethylformamide (40ml) and warmed to 40°C. Sodium cyanide (0.25g,
 5.1 mmol) in water (10ml) was added and the solution stirred at 40°C for 2
 hours. The solution was then cooled to 10°C and bromine (0.82g, 5.1
 mmol) added. Stirring was continued at this temperature for a further 2
 hours. The solution was poured onto water (200ml) and the precipitated
 25 product filtered off, washed with water and dried in a vacuum desiccator.

- 32 -

The product was obtained as a red powder (0.67g, 63%). The dye (50mg) was purified by column chromatography (silica, 1:1 ethylacetate:40-60 petroleum ether) to give the methyl ester containing dye (IIIm) (6mg (12%)) and the free carboxylic acid containing dye (IIn) (3mg (6%)).

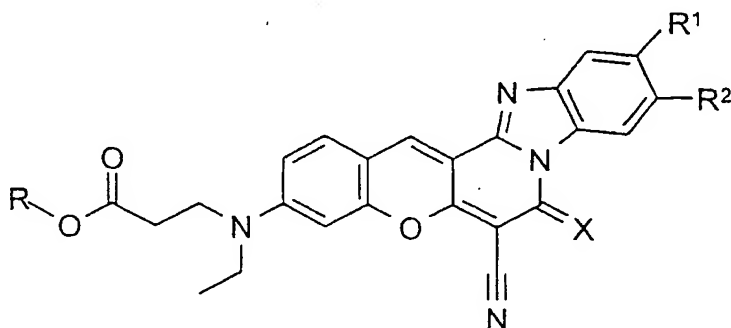
5

Dye (IIIm) λ_{\max} (MeOH) 490nm.Fluorescence (MeOH) λ_{ex} 490nm; λ_{em} 597nm; Stokes Shift 107nm.

^1H NMR (300 MHz, d_6 DMSO) 1.1 (t, 3H), 2.67 (t, 2H), 3.57 (q, 2H),
10 3.62 (s, 3H), 3.73 (t, 2H), 6.82 (s, 1H), 7.01 (d, 1H), 7.21 (m, 2H), 7.26 (t, 2H), 7.70 (m, 3H).

Dye (IIn) λ_{\max} (MeOH) 486nm.15 Fluorescence (MeOH) λ_{ex} 492nm; λ_{em} 597nm; Stokes Shift 105nm.

^1H NMR (300 MHz, d_6 DMSO) 1.09 (t, 3H), 2.67 (t, 2H, $J=6.9$ Hz),
3.57 (q, 2H), 3.73 (t, 2H, $J=6.9$ Hz), 6.82 (s, 1H), 7.01 (d, 1H, $J=9.3$ Hz),
7.21 (m, 2H), 7.26 (t, 2H), 7.70 (m, 3H).

Examples of Red Fluorescent BenzopyranobenzimidazopyridineDyes (III) - Application 3.Example 11.IIIa; R=Me, R¹=R²=H, X=NHIIIb; R=H, R¹=R²=H, X=OIIIc; R=H, R¹=R²=SO₃NaIIId; R=Me, R¹=H, R²=SO₃NaIIIe; R=H, R¹=H, R²=SO₃Na

5

Benzopyranobenzimidazopyridine Dye (IIIa)

A mixture of coumarin (IIa) (2.0g, 5.13 mmol) and malononitrile (0.35g, 5.30 mmol) in 1-butanol was heated at reflux for 4 hours. The mixture was concentrated to ca. 10ml and left to stand overnight during which time the product crystallised out as a dark red solid. The product was filtered off, washed with butanol and dried (1.97g, 87%). The dye (IIIa) was purified by recrystallisation from 1-butanol.

 λ_{\max} (DMF) 541nm.15 Fluorescence (DMF) λ_{ex} 541nm; λ_{em} 574nm; Stokes Shift 33nm.FAB-MS m/z; found 440 (MH⁺); [theoretical (C₂₅H₂₁N₅O₃) 439].

¹H NMR (300 MHz, d₆DMSO) 1.16 (t, 3H, J=6.6 Hz), 2.68 (t, 2H), 3.56 (q, 2H), 3.61 (s, 3H), 3.74 (t, 2H), 6.78 (s, 1H), 6.92 (d, 1H, J=6.9 Hz), 7.37 (m, 2H, J=7.4 Hz), 7.72 (d, 2H, J=8.1 Hz), 8.64 (d, 1H, J=7.2 Hz), 8.68 (s, 1H).

20

Functionalised benzopyranobenzimidazopyridine dye (IIIb)

Imino benzopyranobenzimidazopyridine (IIIa) (200mg, 0.45 mmol) was heated in a mixture of water (20ml) and 10N hydrochloric acid (2ml) at 100°C for 2 hours. The solution was then cooled and the pH adjusted to 5 with the addition of potassium hydroxide solution. The water was removed under reduced pressure and the product washed with methanol, water and then dried. The product (IIIb) was obtained as a purple solid (67mg, 35%).

λ_{\max} (DMF) 545nm.

Fluorescence (DMF) λ_{ex} 545nm; λ_{em} 578nm; Stokes Shift 33nm.

¹H NMR (300 MHz, d₆DMSO) 1.16 (t, 3H), 2.63 (t, 2H), 3.56 (q, 2H), 3.74 (t, 2H), 6.97 (s, 1H), 7.14 (d, 1H), 7.41 (m, 2H), 7.74 (d, 2H), 8.66 (d, 1H), 8.71 (s, 1H).

Sulphonated, functionalised benzopyranobenzimidazopyridine dye (IIIc)

Benzopyranobenzimidazopyridine dye (IIIb) (1g) was stirred in 25% oleum at room temperature for 3 hours, and the solution was poured onto ice and neutralised with NaOH. The solution was evaporated to dryness and a portion of the sodium sulphate/dye mixture (6.5g) was taken and stirred in water (50ml) at room temperature for 30 minutes and then centrifuged. The supernatant was discarded and the washing process repeated with a small amount of water. The resultant solid (60mg) was dried in a vacuum desiccator. This showed a single red fluorescent spot on t.l.c.

λ_{\max} (H₂O) 536nm.

Fluorescence (H₂O) λ_{ex} 536nm; λ_{em} 564nm; Stokes Shift 28nm.

Electrospray (-ve ion)-MS m/z; found 584; [theoretical (C₂₄H₁₆N₄S₂O₁₀) 584].

Using milder sulphonation conditions and purification of the product by HPLC (RP C₁₈, 33% methanol in water), both the methyl ester (IIId)

- 35 -

and the free acid (IIIe) forms of the monosulphonated benzopyranobenzimidazopyridine dye were also isolated.

(IIIId) - Methyl ester

5 MALDI-TOF-MS m/z; found 519 (M^+); [theoretical ($C_{25}H_{20}N_4SO_7$) 520].

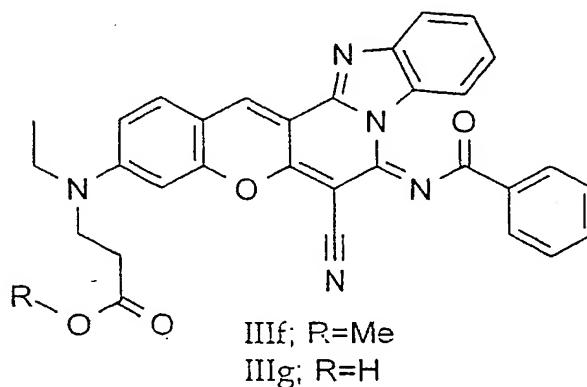
1H NMR (300MHz, d_6 DMSO), 1.17 (t, 3H, $J=6.9$ Hz), 2.71 (t, 2H), 3.37 (q, 2H), 3.63 (s, 3H), 3.80 (t, 2H), 6.78 (s, 1H), 7.03 (d, 1H, $J=7.2$ Hz), 7.63 (d, 1H, $J=8.4$ Hz), 7.71 (d, 1H, $J=8.4$ Hz), 7.84 (d, 1H), 8.85 (s, 1H),
10 8.97 (s, 1H).

(IIIe) - Free acid

MALDI-TOF-MS m/z; found 504 (M^+); [theoretical ($C_{24}H_{17}N_4SO_7$) 505].

15 1H NMR (300MHz, d_6 DMSO), 1.17 (t, 3H), 2.27 (t, 2H), 3.63 (m, 4H), 6.67 (s, 1H), 7.02 (d, 1H), 7.62 (d, 1H, $J=8.4$ Hz), 7.70 (d, 1H, $J=8.4$ Hz), 7.80 (d, 1H, $J=9.6$ Hz), 8.81 (s, 1H), 8.96 (s, 1H).

Example 12.



N-Benzoylimino benzopyranobenzimidazopyridine (IIIIf)

Imino benzopyranobenzimidazopyridine dye (IIIa) (200mg, 0.46 mmol) and benzoyl chloride (1g, 7.12mmol) in pyridine (10ml) were heated at 80°C overnight. The resultant mixture was poured into water (200ml) and

- 36 -

stirred at room temperature for 1 hr and then extracted with dichloromethane. The organic phase was washed with 10% sodium bicarbonate and concentrated *in vacuo*. Purification of the residue by flash column chromatography (Silica: dichloromethane to 20% acetone/dichloromethane) afforded the desired product. The product (III_f) was obtained as a dark red solid (180mg, 73%).

λ_{max} (DMF) 559nm.

Fluorescence (DMF) λ_{ex} 559nm; λ_{em} 596nm; Stokes Shift 37nm.

FAB-MS m/z; found 566 (MNa⁺), 543 (M⁺); [theoretical (C₃₂H₂₅N₄O₄) 543].

N-Benzoylimino benzopyranobenzimidazopyridine (III_g)

Hydrolysis of dye (III_f) under acidic conditions (dilute hydrochloric acid, 90°C, 2h) afforded the free carboxylic acid containing N-benzoylimino-benzopyranobenzimidazopyridine dye (III_g).

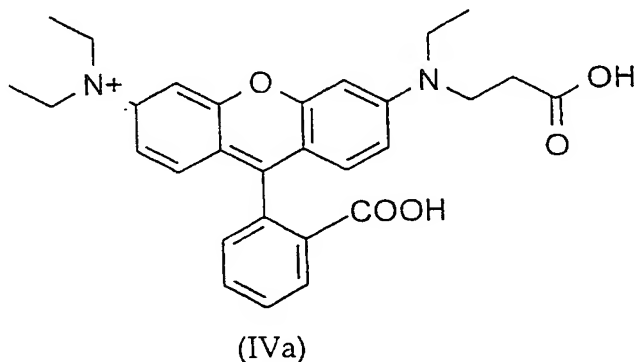
λ_{max} (DMF) 552nm.

Fluorescence (DMF) λ_{ex} 552nm; λ_{em} 580nm; Stokes Shift 28nm.

FAB-MS m/z; found 530 (MH⁺); [theoretical (C₃₁H₂₃N₅O₄) 529].

20 Example of Red-fluorescent Rhodamine Dyes (IV) - Application 4.

Example 13.



- 37 -

Monofunctionalised Rhodamine Dye (IVa)

3-N-Ethyl-N-(2'methoxycarbonylethyl)-aminophenol (Ia) (0.88g, 4mmol), 2-(4'-N,N-diethylamino-2'-hydroxybenzoyl)benzoic acid (1.13g, 4mmol) and concentrated sulphuric acid (15ml) were stirred together at
5 room temperature for 24hrs. The reaction mixture was then slowly neutralised with sodium hydroxide solution (20%) until the rhodamine dye started to crystallise out of solution. The dye was filtered off and dried. The dye was purified by column chromatography (silica; 1:1 chloroform/methanol, finally dissolving dye in chloroform and filtering to
10 remove the silica). The product (IVa) was obtained as a magenta powder (0.02g, 1%).

λ_{\max} (MeOH) 544nm.

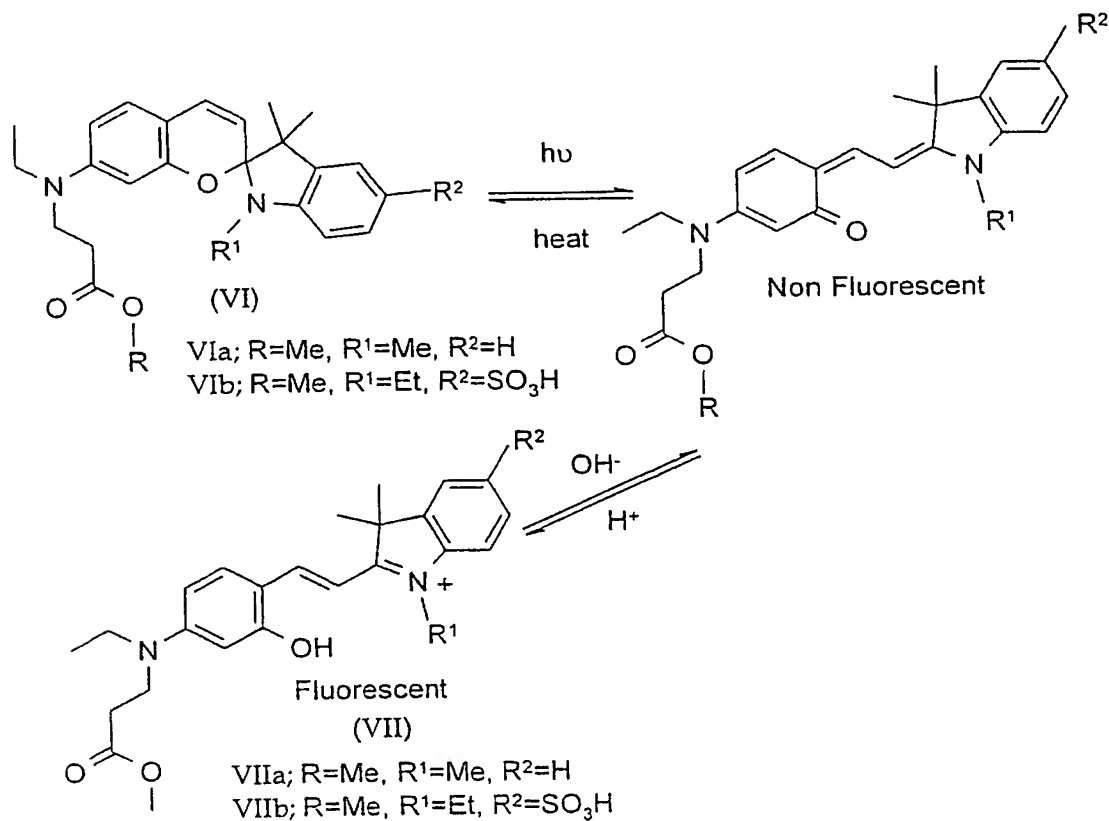
Fluorescence (MeOH) λ_{ex} 547nm; λ_{em} 570nm; Stokes Shift 23nm.

FAB-MS; found 487; [theoretical ($\text{C}_{29}\text{H}_{31}\text{N}_2\text{O}_5$) 487].

15 ^1H NMR (300 MHz, CD_3OD) 1.28 (t, 9H, $J=6.0$ Hz), 2.53 (t, 1H, $J=7.2$ Hz), 3.66 (m, 6H, $J=7.5$ Hz), 3.84 (t, 1H, $J=7.5$ Hz), 7.00 (m, 4H), 7.26 (m, 3H), 7.62 (m, 2H), 8.08 (d, 1H, $J=4.5$ Hz).

Examples of pH Sensitive Photochromic Dyes (VI) and basic dye forms (VII) - Application 6.

Example 14.



- 5 4-(N-ethyl-N-(2'-methoxycarbonyl)ethyl)-amino)benzaldehyde (1g, 0.40 mmol), Fischer's base (0.6g, 0.40 mmol), triethylamine (0.5ml) and ethanol (10ml) were heated together at reflux for 4 hours. On cooling the product crystallised out of solution and was filtered off, washed with methanol and dried. The product was isolated in the colourless spiro form (VIa) (0.7g, 50%). It was noted that the non-fluorescent merocyanine form (VIa open) could be switched to the fluorescent basic dye form (VIIa) by adding acid to a solution of dye in methanol.

Merocyanine Form (VIa)

15 λ_{\max} (MeOH) 550nm, E_{\max} 37,677 l.mol⁻¹.cm⁻¹.

- 39 -

Fluorescence (MeOH) λ_{ex} 563nm; λ_{em} 574nm; Stokes Shift 11nm,
- negligible fluorescence.

Basic Form (VIIa)

5 λ_{max} (MeOH) 544nm, E_{max} 71,968 l.mol⁻¹.cm⁻¹.

Fluorescence (MeOH) λ_{ex} 569nm; λ_{em} 581nm; Stokes Shift 12nm,

¹H NMR Basic dye (VIIa), (300MHz, CD₃OD), 1.24 (t, 3H, J=6.9 Hz),
1.76 (s, 6H), 2.70 (t, 2H, J=7.2 Hz), 3.56 (q, 2H, J=6.9 Hz), 3.69 (s, 3H),
3.78 (t, 2H, J=6.9 Hz), 3.83 (s, 3H), 6.18 (s, 1H), 6.52 (d, 1H, J=9.3 Hz),
10 7.17 (broad d) 7.41 (m, 4H), 7.81 (d, 1H, J=9.9 Hz), 8.57 (d, 1H, J=14.7
Hz).

Photochromic Dye (VIb)

2- Acetoxy-4-(N-ethyl-N-(2'methoxycarbonylethyl)-amino)-
15 benzaldehyde (Ie) (0.5g, 1.7mmol), 1-ethyl-2,3,3-trimethyl-indolium-5-
sulphonate (0.46g, 1.7mmol), triethylamine (0.25ml) and ethanol (10ml)
were heated at reflux for 5 hours. On cooling the ethanol was removed
under reduced pressure to leave a magenta tar which was triturated with
ether to give a sticky magenta solid. The dye was purified by column
20 chromatography (silica; 30% methanol in ethyl acetate). The product (VIb)
was obtained as a purple solid (0.11g, 11.4%). In order to convert dye (VIb)
to the basic dye form (VIIb) a small sample of the dye was dissolved in
methanol containing hydrochloric acid. The dye was then reprecipitated by
adding ether, filtered off and dried.

25 (VIIb) λ_{max} (MeOH) 554nm.

Fluorescence (VIIb) (MeOH) λ_{ex} 544nm; λ_{em} 577nm;

Stokes Shift 33nm.

¹H NMR (VIIb) (300 MHz d₆DMSO), 1.14 (broad, 3H), 1.34 (broad,
3H), 1.69 (s, 6H), 2.67 (broad, 2H), 3.48 (broad, 2H), 3.62 (s, 3H), 3.70

- 40 -

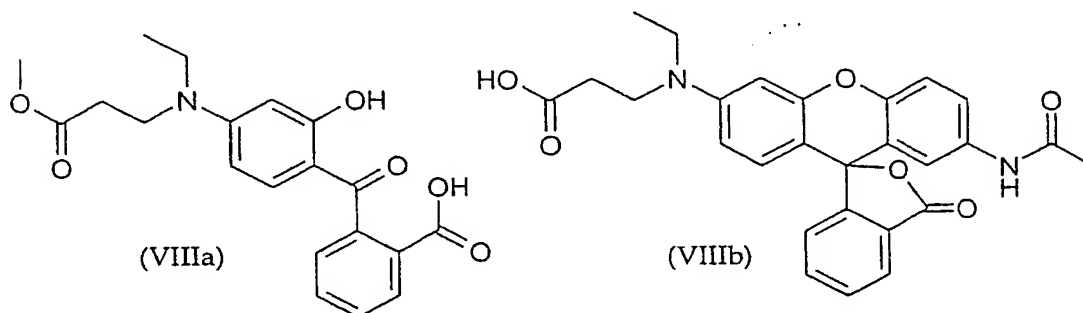
(broad, 2H), 4.38 (broad), 6.29, (s, 1H), 6.53 (d, 1H), 7.23 (d, 1H), 7.59 (d, 1H), 7.71 (d, 1H), 7.90 (s, 1H), 8.01 (d, 1H), 8.41 (d, 1H).

Example of pH sensitive green-black floran dye (XIII) -

Application 7

5

Example 15.



Green-black floran dye (VIIIb)

Phthalic anhydride (2.66g, 0.018mol) was added to a stirred solution of 3-N-ethyl-N-(2'methoxycarbonyl-ethyl)-aminophenol (Ia) (4g, 0.018mol) in 5ml chlorobenzene, the resulting solution was then heated at 100°C for 8 hours. The chlorobenzene solvent was removed on a rotary evaporator yielding a red tar, which TLC showed to contain significant quantities of unreacted material (yield 6.57g). This was purified by dissolving the solid in a dilute solution of sodium hydroxide, followed by extraction with dichloromethane. The product remained in the aqueous phase and was then extracted into dichloromethane after acidifying the solution. Evaporation of the solvent gave (VIIIa) (1.2 g; 18%).

Intermediate (VIIIa) (0.3g, 1.1mmol) was dissolved in conc. H₂SO₄ (4ml) and 3-dimethylaminophenol (0.15g, 1.1mmol) was added in portions over 15 minutes. The solution was then stirred at room temperature for 65 hours. TLC analysis of the solution showed it to contain 3 components.

The acidic solution (~3ml) was poured onto a 20g water / ice mixture and slowly neutralised with concentrated sodium hydroxide, and the

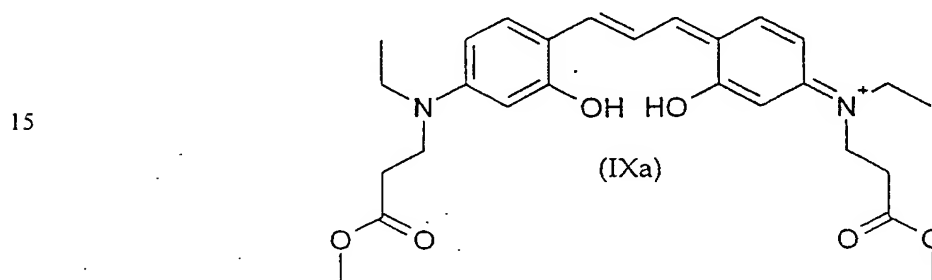
- 41 -

resultant fluoran lactone species was extracted at ~ pH 6 into 2x100ml portions of dichloromethane. The extracts were dried and evaporated under reduced pressure. This gave (VIIb) in 1% yield. The visible absorption spectrum of the dye showed $\lambda_{\max} = 530\text{nm}$ under acid condition, the peak disappearing (reversibly) under basic conditions due to lactone formation.

The mass spectrum of the product was consistent with the assigned structure (FAB: found $m/Z = 473$, with weaker peak at $m/Z = 495$; $\text{C}_{27}\text{H}_{24}\text{N}_2\text{O}_6$ requires $M+1 = 473$, $M+23 = 495$).

10 Example of Styryl hemi-Cyanines (IX) - Application 8.

Example 16.



Styryl hemi-

Cyanine Dye

20 (IXa)

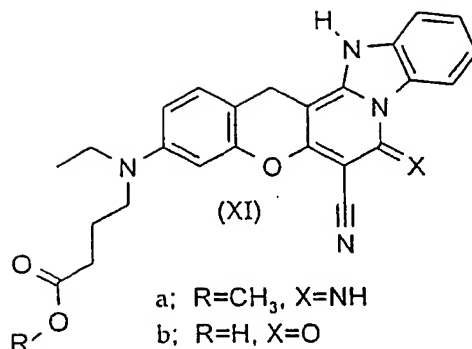
3- Anilinoacrylaldehyde anil (0.25g, 1.1mmol) and acetic anhydride (1ml) were heated together at 40°C for 30mins. 3-N-Ethyl-N-(2'methoxycarbonyl)ethyl)-aminophenol (Ia) (0.5g, 2.3mmol) and acetic acid (5ml) were then added and the mixture was warmed to 40°C and stirred for 1hr. On cooling ether (100ml) was added and the solvents were then decanted off to leave a cyan coloured tar.

λ_{\max} (MeOH) 660nm.

Electrospray-MS (+ve ion); found 483 (M^+); [theoretical ($\text{C}_{27}\text{H}_{35}\text{N}_2\text{O}_6$) 484].

30 Examples of Leuco dyes for use as enzyme substrates

- Application 9.

Example 17.**Benzopyranobenzimidazopyridine leuco dye (a)**

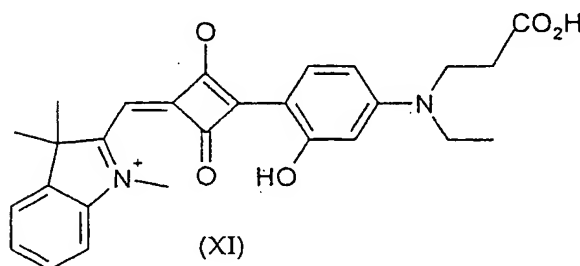
10 Sodium borohydride (excess) was added to a solution of benzopyranobenzimidazopyridine dye (IIIa) (0.5g) in dichloromethane/methanol (60/10ml). After 10 mins, water (50ml) and 10N hydrochloric acid (1 drop) was added and the mixture was stirred for a

15 further 10 mins in order to destroy the excess sodium borohydride. The pale yellow solid (a) which formed was filtered off and dried overnight *in vacuo*.

Benzopyranobenzimidazopyridine leuco dye (b)

20 Sodium borohydride (at least 5 fold excess) was added to a solution of the benzopyranobenzimidazopyridine dye (IIIb) (1g) in methanol (100ml). After the effervescence had ceased (ca. 10 min) the solution was concentrated *in vacuo* and then dried overnight *in vacuo* to afford the crude *leuco* dye (b), 1.35g.

25 In both of the above cases treatment of a solution of the *leuco* dye in DMF with a solution of chloranil in DMF regenerated the colour (and hence the absorption spectrum) of the parent dye.

Examples of squarylium (XI) - Application 11.Example 18.Unsymmetrical squarylium dye (XI)

Dimethyl squarate (0.50 g) and Fischer's base (0.61) were dissolved in methanol (15 ml) containing sodium methoxide (0.3g) and the solution stirred at 30 °C for 5 hours. To the solution was added n-butanol

15 (40 ml), toluene (20 ml), 3-N-ethyl-N-(2'methoxycarbonylethyl)-aminophenol (Ia) (0.79 g) and 5 drops of concentrated hydrochloric acid, and the mixture heated under reflux for 4 hours. The solvent was then removed under reduced pressure and the residue chromatographed over silica gel using dichloromethane with increasing proportions of acetone as

20 eluent. When the first blue band had been eluted the second blue band was eluted with methanol, affording, after evaporation of the solvent, dye (XI) (0.45 g), which was characterised by mass spectrometry.

λ_{max} (MeOH) 628nm.

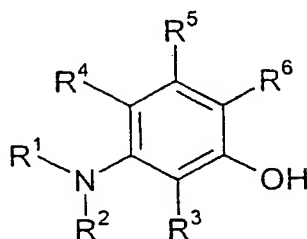
Fluorescence (MeOH) λ_{ex} 628nm; λ_{em} 648nm; Stokes Shift 18nm.

25

CLAIMS

5

1. Use of a compound having the formula



(I)

10

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or a branched chain, and R^b is carboxy including acid, salt, ester including N-hydroxysuccinimide, activated ester or amide,

R^2, R^3, R^4 and R^5 are the same or different and each is: H; or

15

$C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ where R^a and R^c are herein defined,

20

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

R^6 is H or $-CR^9O$ or NO ,

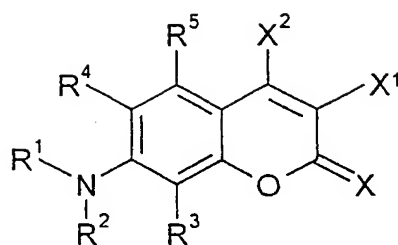
R^9 is H, $C_1 - C_6$ alkyl, phenyl, or R^aR^c wherein R^a and R^c are

25

herein defined,

to make a dye selected from

- 45 -

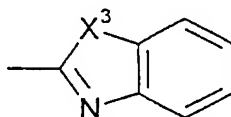


(II)

where X is O, NH or =NCOAr

X¹ is H, CN, CHO, CH=N⁺(R^dR^e), NO, COOH, COOR^f,

5 CONR^gR^h, C₁ – C₁₀ alkyl, aralkyl or aryl or



where X³ is NH, N-R^k, O or S, wherein X¹ is either
unsubstituted or substituted by: a group to modify solubility; or a group to
modify electronic and/or spectral properties; or a functional group for
10 linking to another component,

R^d and R^e are alkyl, aryl, aralkyl,

R^f, R^g, R^h and R^k are R^aR^c wherein R^a and R^c are herein
defined,

15 X² is H, CN, ORⁱ, Cl, Br, alkyl or aryl which maybe
unsubstituted or substituted by: a group to modify solubility; or a group to
modify electronic and/or spectral properties; or a functional group for
linking to another component;

where Rⁱ is MeC₆H₄SO₂⁻, CH₃SO₂⁻, P=O(OR^j)₂

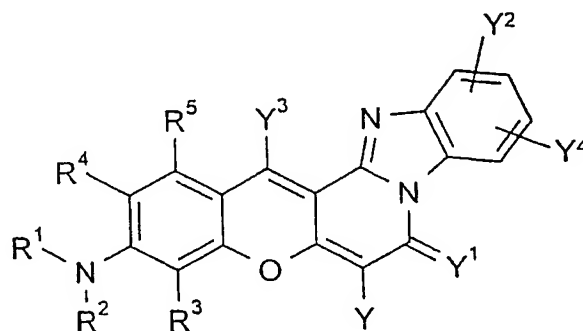
20 R^j is alkyl, aryl, aralkyl

X¹ and X² may contain atoms which are linked to form a
carbocyclic, heterocyclic, aliphatic or aromatic ring system which may be
substituted or unsubstituted with a group to modify electronic and/or
spectral properties and/or a group to modify solubility properties and/or a

- 46 -

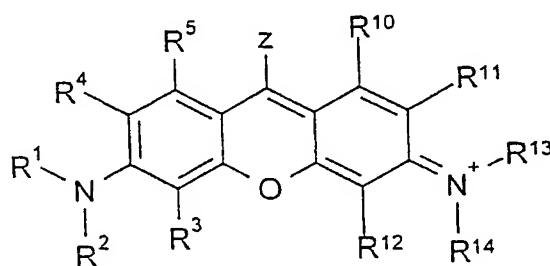
functional linking group.

provided that, when X is O, then X^1 is not H, CN, or $C_1 - C_{10}$ alkyl, aralkyl or aryl; and provided that when X is O, then X^3 is not S;



(III)

- 5 where Y is CN or CONH₂ or CH₂NH₂,
 Y^1 is NH or O or =NCOAr,
 Y^2 and Y^4 are: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to
 10 modify electronic and/or spectral properties, or a functional linking group; or
 - $R^a R^c$ wherein R^a and R^c are herein defined,
 Y^3 is H or CN;



(IV)

- 15 where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic

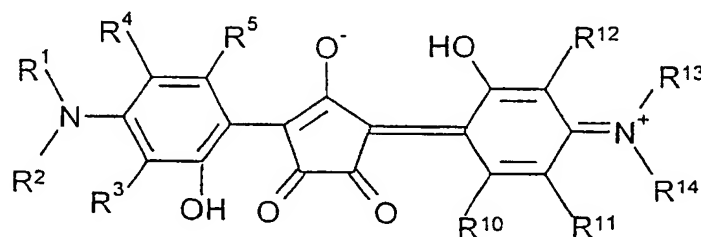
- 47 -

and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

R^{10} and R^{11} ; and/or R^{13} and R^{14} ; and/or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic,
 5 aromatic or aliphatic which is unsubstituted or substituted as above,

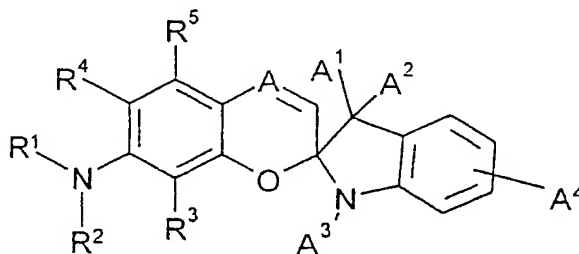
Z is aryl which is unsubstituted or substituted by: a group to modify solubility; or a group to modify electronic and/or spectral properties; or a functional group for linking to another component, provided that Z is not 2-carboxy-3,4,5,6-tetrachloro phenyl;

10



(V)

provided that $NR^{13}R^{14}$ is different from NR^1R^2 ;



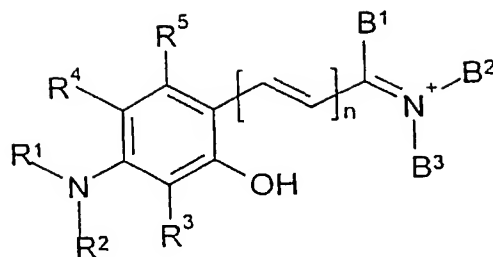
(VI)

15

where A is N or CH

A^1 , A^2 , A^3 and A^4 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral
 20 properties, or a functional linking group; or $-R^aR^c$ where R^a and R^c are

herein defined;

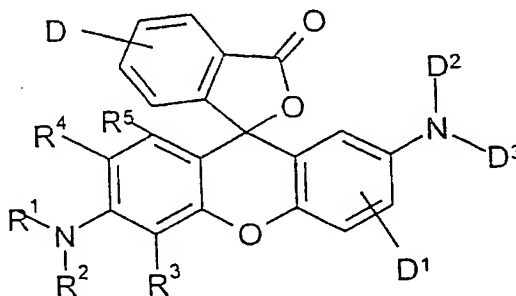


(VII)

where n is 0, 1 or 2, B¹ is H, C₁-C₁₀ alkyl, aryl or aralkyl either
 5 unsubstituted or substituted by a group to modify solubility or a group to
 modify electronic and/or spectral properties, or a functional linking group,
 Cl, Br or F,

B² and B³ are the same or different and are selected from C₁-
 C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to
 10 modify solubility or a group to modify electronic and/or spectral properties,
 or a functional linking group,

B¹ and B² and/or B¹ and B³ and/or B² and B³ may be linked to
 form an extended ring system, carbocyclic or heterocyclic, aromatic or
 aliphatic which is unsubstituted or substituted by a group to modify
 15 solubility, or a group to modify electronic and/or spectral properties, or a
 functional linking group;

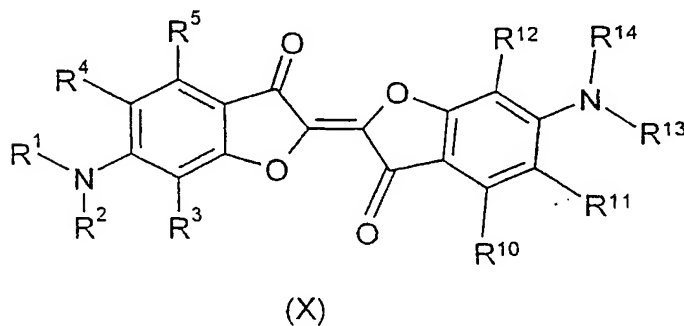
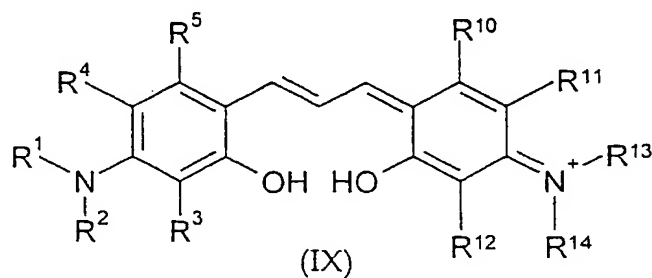


(VIII)

where D, D¹, D² and D³ are the same or different and each is:

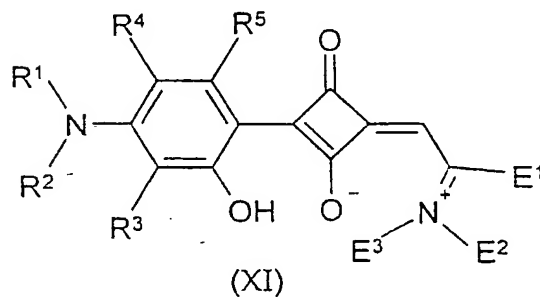
- 49 -

H; or C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or – R^aR^c where R^a and R^c are
 5 herein defined;



10

and

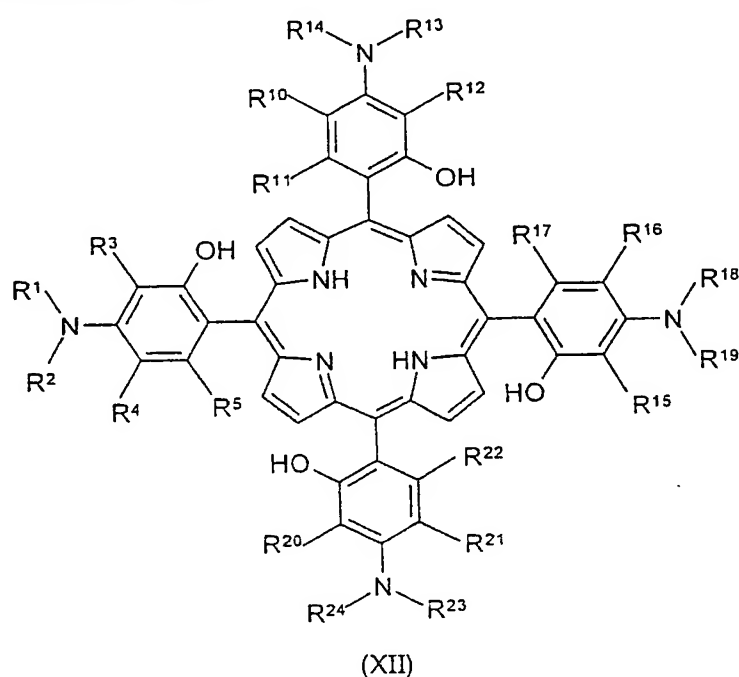


where E¹ is H, C₁-C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group, Cl, Br or F,
 15 E² and E³ are the same or different and are selected from C₁-

- 50 -

C₁₀ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group,

- E¹ and E² and/or E¹ and E³ and/or E² and/or E³ may be linked
 5 to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted by a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; and



with or without a central metal ion,

- 10 where R¹⁵, R¹⁶, R¹⁷, R¹⁸, R¹⁹, R²⁰, R²¹, R²², R²³ and R²⁴ are the same or different and each is: H; or C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or
 15 – R^aR^c wherein R^a and R^c are herein defined,

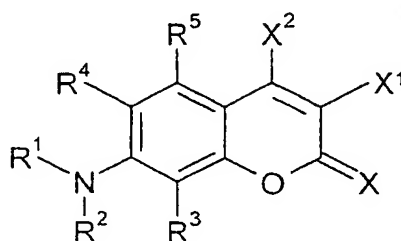
R¹⁶ and R¹⁷; and/or R²¹ and R²²; and/or R¹⁵ and R¹⁸ and/or R¹⁶ and R¹⁹; and/or R²⁰ and R²³; and/or R²¹ and R²⁴ may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic

- 51 -

which is unsubstituted or substituted as above;

or a leuco-dye analogue of the said dye.

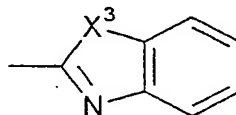
5 2. A dye having the formula:



(II)

where X is O, NH or =NCOAr

X¹ is H, CN, CHO, CH=N⁺(R^dR^e), NO, COOH, COOR^f,
 10 CONR^gR^h, C₁ – C₁₀ alkyl, aralkyl or aryl or



where X³ is NH, N-R^k, O or S, wherein X¹ is either
 unsubstituted or substituted by: a group to modify solubility; or a group to
 modify electronic and/or spectral properties; or a functional group for
 15 linking to another component,

R^d and R^e are alkyl, aryl, aralkyl;

R^f, R^g, R^h and R^k are R^aR^c wherein R^a and R^c are herein
 defined,

20 X² is H, CN, ORⁱ, Cl, Br, alkyl or aryl which maybe
 unsubstituted or substituted by: a group to modify solubility; or a group to
 modify electronic and/or spectral properties; or a functional group for
 linking to another component;

where Rⁱ is MeC₆H₄SO₂-, CH₃SO₂-, P=O(OR^j)₂

- 52 -

R^j is alkyl, aryl, aralkyl

X^1 and X^2 may contain atoms which are linked to form a carbocyclic, heterocyclic, aliphatic or aromatic ring system which may be substituted or unsubstituted with a group to modify electronic and/or spectral properties and/or a group to modify solubility properties and/or a functional linking group.

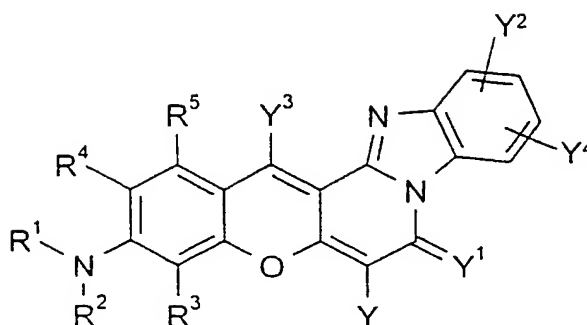
provided that, when X is O, then X^1 is not H, CN, or $C_1 - C_{10}$ alkyl, aralkyl or aryl; and provided that when X is O, then X^3 is not S;

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

3. A dye having the formula



(III)

5 where Y is CN or CONH₂ or CH₂NH₂,

Y¹ is NH or O or =NCOAr.

Y² and Y⁴ are: H; or C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or - R^aR^c wherein R^a and R^c are herein defined,

Y^3 is H or CN:

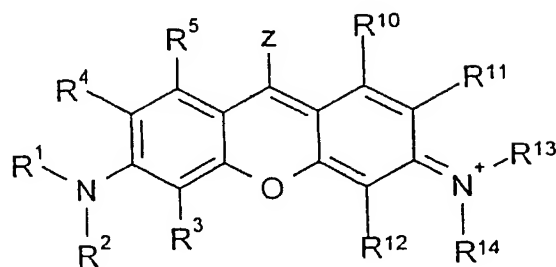
where R¹ is -R^aR^b, where R^a is C₁ - C₁₀ alkylene or
aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur
atoms in a straight or branched chain, and R^b is carboxy including acid,
salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or R⁴ – R⁵ and/or R² – R⁴ and/or R² – R³ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic

which is unsubstituted or substituted as above.

4. A dye having the formula



(IV)

5

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

10

R^{10} and R^{11} ; and/or R^{13} and R^{11} ; and/or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

15

Z is aryl which is unsubstituted or substituted by: a group to modify solubility; or a group to modify electronic and/or spectral properties; or a functional group for linking to another component, provided that Z is not 2-carboxy-3,4,5,6-tetrachloro phenyl;

20

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

25

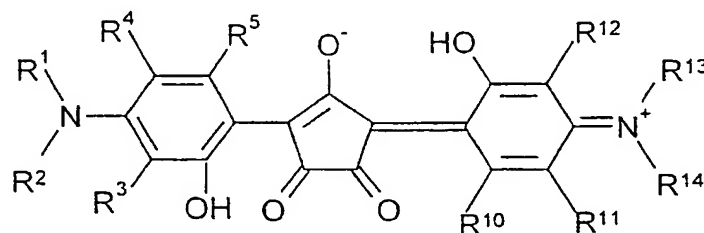
R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral

- 55 -

properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an
 5 extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic
 which is unsubstituted or substituted as above.

5. A dye having the formula



(V)

provided that $NR^{13}R^{14}$ is different from NR^1R^2 ;

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and
 each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4
 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c
 15 where R^c is a group to modify solubility, or a group to modify electronic
 and/or spectral properties; or a functional linking group; or $-R^aR^c$ wherein
 R^a and R^c are herein defined,

R^{10} and R^{11} ; and/or R^{13} and R^{14} ; and/or R^{12} and R^{14} may be
 linked to form an extended ring system, carbocyclic or heterocyclic,
 20 aromatic or aliphatic which is unsubstituted or substituted as above,

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or
 aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur
 atoms in a straight or branched chain, and R^b is carboxy including acid,
 salt, ester including N—hydroxysuccinimide, activated ester or amide,

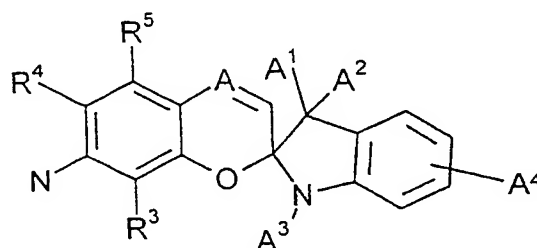
25 R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or
 $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen,

nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

5 or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

6. A dye having the formula

10



(VI)

where A is N or CH

A^1, A^2, A^3 and A^4 are the same or different and each is: H; or

15 C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or – R^aR^c are herein defined;

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or

20 aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2, R^3, R^4 and R^5 are the same or different and each is: H; or

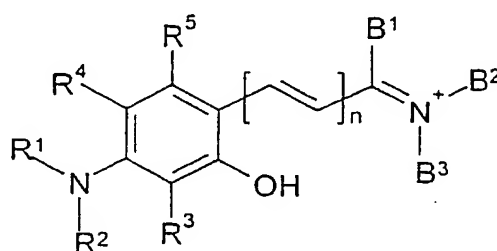
25 C₁ – C₁₀ alkyl or aralkyl which may optionally contain 1 to 4 oxygen,
nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is
a group to modify solubility, or a group to modify electronic and/or spectral

- 57 -

properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic
 5 which is unsubstituted or substituted as above.

7. A dye having the formula



(VII)

10 where n is 0, 1 or 2, B^1 is H, C_1 - C_{10} alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group, Cl, Br or F,

B^2 and B^3 are the same or different and are selected from C_1 -
 15 C_{10} alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group,

B^1 and B^2 and/or B^1 and B^3 and/or B^2 and B^3 may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or
 20 aliphatic which is unsubstituted or substituted by a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group;

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur
 25 atoms in a straight or branched chain, and R^b is carboxy including acid,

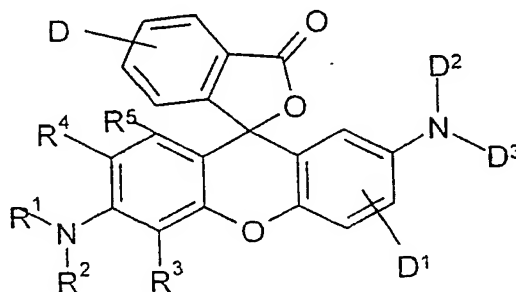
salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

8. The dye of claim 7 which is in a merocyanine non-fluorescent form or in a basic fluorescent form.

9. A dye having the formula



(VIII)

where D, D^1 , D^2 and D^3 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atom in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ where R^a and R^c are herein defined;

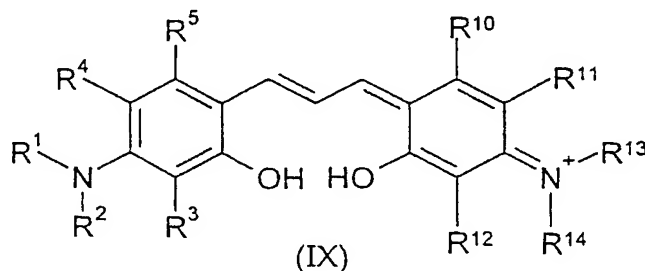
where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

- 59 -

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

10 10. A dye having a formula



and

15

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

R^{10} and R^{11} ; and/or R^{13} and R^{11} ; and/or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

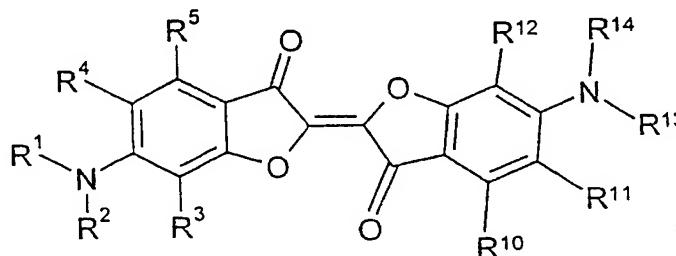
25 where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur

atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

11. A dye having the formula



(X)

15

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

20

R^{10} and R^{11} ; or R^{13} and R^{11} ; or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

25

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur

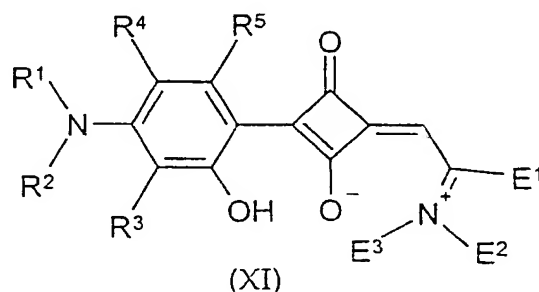
- 61 -

atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

12. A dye having the formula



where E^1 is H, $C_1 - C_{10}$ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group, Cl, Br or F,

E^2 and E^3 are the same or different and are selected from $C_1 - C_{10}$ alkyl, aryl or aralkyl either unsubstituted or substituted by a group to modify solubility or a group to modify electronic and/or spectral properties, or a functional linking group,

E^1 and E^2 and/or E^1 and E^3 and/or E^2 and/or E^3 may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted by a group to modify solubility, or a group to modify electronic and/or spectral properties, or a

functional linking group;

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

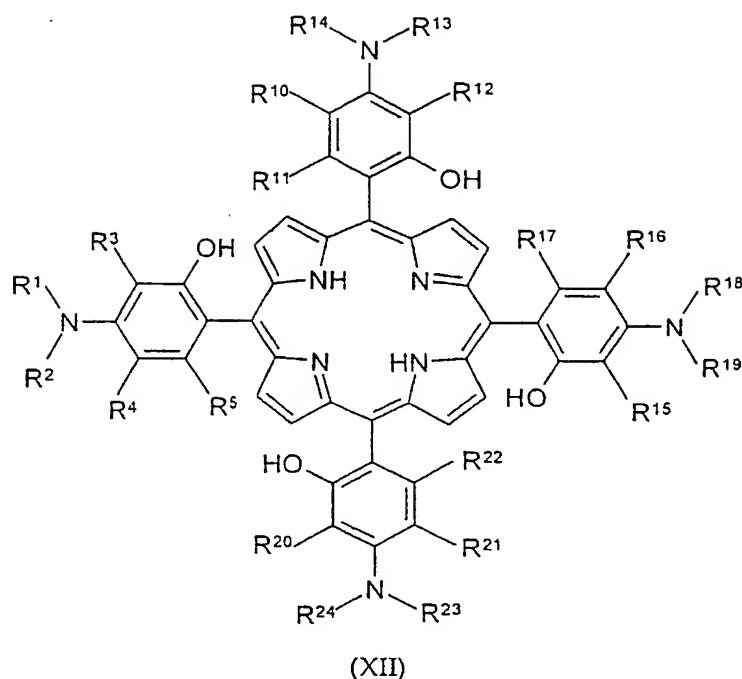
5 R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are
10 herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

15

13. A dye having the formula

- 63 -



with or without a central metal ion,

where R^{15} , R^{16} , R^{17} , R^{18} , R^{19} , R^{20} , R^{21} , R^{22} , R^{23} and R^{24} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

R^{16} and R^{17} ; and/or R^{21} and R^{22} ; and/or R^{15} and R^{18} and/or R^{16} and R^{19} ; and/or R^{20} and R^{23} ; and/or R^{21} and R^{24} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

where R^{10} , R^{11} , R^{12} , R^{13} and R^{14} are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

- 64 -

R^{10} and R^{11} ; and/or R^{13} and R^{11} ; and/or R^{12} and R^{14} may be linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

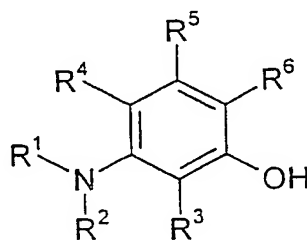
where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above.

14. A leuco-dye or reduced dye analogue of the dye as claimed in any one of claims 2 to 13.

15. A method of making a compound having the formula



(I)

where R^1 is $-R^aR^b$, where R^a is $C_1 - C_{10}$ alkylene or aralkylene which may optionally contain 1 to 4 oxygen, nitrogen or sulphur

- 65 -

atoms in a straight or branched chain, and R^b is carboxy including acid, salt, ester including N—hydroxysuccinimide, activated ester or amide,

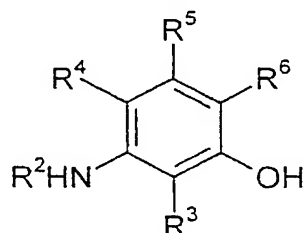
R^2 , R^3 , R^4 and R^5 are the same or different and each is: H; or $C_1 - C_{10}$ alkyl or aralkyl which may optionally contain 1 to 4 oxygen, nitrogen or sulphur atoms in a straight or branched chain; or R^c where R^c is a group to modify solubility, or a group to modify electronic and/or spectral properties, or a functional linking group; or $-R^aR^c$ wherein R^a and R^c are herein defined,

or $R^4 - R^5$ and/or $R^2 - R^4$ and/or $R^2 - R^3$ are linked to form an extended ring system, carbocyclic or heterocyclic, aromatic or aliphatic which is unsubstituted or substituted as above,

R^6 is H or $-CR^9O$ or NO ,

R^9 is H, $C_1 - C_6$ alkyl or phenyl,

which method comprises reacting



with GG^1

where G is $CHR=CR-$ where R is H or $C_1 - C_4$ alkyl

and G^1 is an electron donating group for example CN or

carboxy where the carboxy group is acid, salt, ester or amide.

16. A method as claimed in claim 15 wherein GG^1 is a $C_1 - C_4$ alkyl acrylate or methacrylate.

17. A complex of a dye according to any one of claims 2 to 14 with a biomolecule, wherein the compound is covalently linked to the biomolecule through R^1 .

- 66 -

18. Use of a dye according to any one of claims 2 to 14 as a donor or acceptor in a resonance energy transfer system.
- 5 19. Use of a dye according to any one of claims 2 to 14 in fluorescence polarisation assays